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# *Bacillus thuringiensis* and Related Insect Pathogens

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## INTRODUCTION

The group of microorganisms pathogenic for insects is varied and diverse. Among these are a broad range of viruses, bacteria, and fungi (Table 1). Each of these subgroups is composed of a spectrum of organisms that vary in their mode of infection, site of replication, and mechanism of pathogenicity. While some pathogens exhibit rather wide host ranges, many have their preferred targets among certain insect species as well as selective pathogenicity for larval or adult life stages. This spectrum probably reflects the overlap in natural habitats of particular microorganisms and insects, the mechanism of pathogenicity (nature of toxic substances produced), and the needs of the pathogen.

Although mycoplasma-like organisms have been observed to infect insects, their etiological role in disease has not been elucidated (21). A number of rickettsia cause disease in insects; others exist in commensural or symbiotic relationship with their arthropod hosts (21, 101). The group of entomopathogenic viruses includes forms with both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) genomes. In general, insect viruses are characterized by a high degree of host specificity, although individual viruses with rather broad host ranges are known. The initial site of infection of many of these viruses is the midgut, and many are able to spread rapidly from cell to cell. Entomopathogenic species of fungi, often with a broad host range, are found among the phycomycetes, ascomycetes, basidiomycetes, and fungi imperfecti. Fungal pathogens of insects are unique in their ability to infect by penetrating sclerotized, chitinous insect cuticle.

The group of entomopathogenic bacteria includes species with the ability to infect uncompromised healthy insects and also a large number of opportunistic pathogens which multiply rapidly if they gain access to the hemocoel of stressed insect hosts through wounds or following infection. An interesting example of this latter group is the bacterium *Xenorhabdus nematophilus*, which is harbored in the alimentary tract of the entomopathogenic nematode *Steinernema feltiae*. The nematode acts as a syringe to inoculate susceptible insect larvae with the bacterium, which then multiplies and kills the insect host. The nematode feeds on the bacteria and multiplies in the insect carcass, and progeny nematodes exit carrying bacterial inocula to infect additional insects. Clostridial pathogens differ from other species that infect healthy insects in that these bacteria multiply only in the gut and do not invade the hemocoel. They are virulent for specific insect hosts and apparently kill without producing highly poisonous toxins (60).

The major species of bacteria with mechanisms to infect and kill uncompromised, healthy insects are sporeforming bacilli. For these species, the hemolymph of insect larvae is an excellent nutritional environment for bacterial proliferation, and sometimes for sporulation (24, 54). In fact, the

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TABLE 1. Microorganisms pathogenic to insects

| Group | Representative species | Susceptible insects | Reference |
|---|---|---|---|
| Viruses | *Autographa californica* nuclear polyhedrosis virus | Lepidoptera, Hymenoptera, Coleoptera, Diptera, Neuroptera, Orthoptera, Trichoptera, Hemiptera, and others | 64, 76, 93, 141 |
| Rickettsiae | *Rickettsiella melolontha* | Coleoptera, Diptera, Orthoptera | 101 |
| Fungi | *Beauvaria bassiana, Metarrhizium anisopliae* | Lepidoptera, Homoptera, Hymenoptera, Coleoptera, Diptera | 145, 165, 188 |
| Bacteria | *Bacillus popillae* | Scarabaeid beetles | 19, 24, 25 |
| | *B. thuringiensis* | Lepidoptera, Diptera | 24, 25 |
| | *B. sphaericus* | Mosquitoes | 24, 25 |
| | *Clostridium malacosome* | *Malacosoma* spp. (tent caterpillars) | 16 |
| | *Pseudomonas aeruginosa* | Opportunistic pathogen with susceptible species in most major insect taxa | 17 |
| | *Xenorhabdus nematophilus* | Insects susceptible to the nematode *Steinernema feltiae* (wide host range) | 143, 144 |

hemolymph may be one of the rare environments in which soil bacteria can reach sufficiently high concentrations so that genetic exchange via cell mating may occur (29, 71, 73, 129).

Among the sporeforming bacilli, there are major groups (*Bacillus thuringiensis* and *B. sphaericus*) which produce protoxins during sporulation. These proteins are deposited as parasporal inclusions and, in some cases, are also found on the surface of the spore. When the inclusion (or spore) is ingested by susceptible larvae (i.e., Lepidoptera and Diptera), the protoxin is solubilized in the alkaline, reducing environment of the midgut. The midguts of these larvae also contain proteases necessary to convert the protoxins to toxins and perhaps receptors on the surface of midgut epithelial cells to which the toxins (or protoxins) bind to initiate their action (63, 86, 100).

It is quite possible that the range of insects susceptible to sporeforming bacilli is much broader than indicated in Table 1. A recently described isolate, *B. thuringiensis* subsp. *tenebrionis*, is toxic to some coleopteran larvae (102). Members of this order may have acidic rather than alkaline guts (L. Murdock and P. Dunn, unpublished data) so the type of toxin produced by this subspecies and its mode of action may be unique.

Currently there is an enhanced interest in the parasporal body-forming bacilli because of the potential for manipulating cloned protoxin genes. There have been periodic reviews of these organisms (18, 21, 30, 52, 146, 161), but recent advances in the plasmid biology, genetics, and cloning have provided new insights and approaches for understanding the basis for toxicity and the regulation of synthesis of inclusion proteins. We have included several sporeforming bacilli in this review, but the primary focus is on those forming parasporal inclusions.

## PRACTICAL CONSIDERATIONS

Of the bacteria pathogenic to insects, the sporeforming bacilli are viewed as having the highest potential for use in the management of insect pest populations, and these organisms have received the greatest commercial attention (52, 53, 56). There are currently 410 registered formulations of *B. thuringiensis* and 6 registered formulations of *B. lentimorbus* and *B. popillae* approved for use in the United States against insect pests (according to the National Pesticide Information Retrieval System, Purdue University, West Lafayette, Ind.). While no formulations of *B. sphaericus* have as yet been registered, pilot batches of a commercial product containing strain 1593 have been evaluated (24). Many approved formulations of *B. thuringiensis* contain additional active ingredients including both fungicides and chemical insecticides and, as a result, these products are registered for use against a spectrum of target pests far exceeding the larvae of lepidopteran and dipteran species susceptible to the spores and crystals of the bacterium. Use of the milky spore disease organisms *B. lentimorbus* and *B. popillae* is limited to their scarabaeid coleopteran hosts. The potential targets of *B. sphaericus* are the larvae of certain mosquito species against which this bacterium is more efficacious than the *israelensis* subspecies of *B. thuringiensis* due to either greater potency or the capacity to replicate in some environments (24).

The use of these bacteria for practical pest control has both advantages and problems. Their common advantage is the production of stable spores which are readily formulated for use in conventional pest control application equipment and which are remarkably safe for humans, other mammals, and nontarget fauna (24). Infections of the milky disease organisms may spread and persist in natural environments and thus may only need be applied once for long-term control. At present, however, spores of *B. lentimorbus* and *B. popillae* can only be produced in vivo, using diseased insects. Under certain conditions, disease in mosquito populations caused by *B. sphaericus* may also spread and persist in aquatic environments, making this organism attractive for control of mosquitoes in inaccessible habitats, such as water in tree holes, where repeated application is extremely labor intensive (24).

On the other hand, *B. thuringiensis* rarely induces epizootics except in cases where insects are in confined or crowded areas such as bee hives, insect-rearing facilities, or stored grain bins (56). Further, preparations of *B. thuringiensis* spores and parasporal crystals applied to foliage are washed off by rain and may be inactivated by sunlight so only relatively short-term protection from pest populations is obtained from single applications. Thus, *B. thuringiensis* must be used as a conventional insecticidal gut poison and applied repeatedly for long-term protection of agricultural crops. While isolates of *B. thuringiensis* with broad-spectrum toxicity for lepidopteran pests are available (i.e., the HD-1 isolate of *B. thuringiensis* subsp. *kurstaki*), no single isolate is active against all pest species. A major problem with the use of *B. thuringiensis* subsp. *israelenis*

TABLE 2. Plasmid content and location of protoxin genes in *B. thuringiensis* subspecies

| Subspecies | | | Plasmids: no. (size range in MDa) | Location of protoxin gene (size of plasmid in MDa or chromosomal) by: | | Reference(s) |
|---|---|---|---|---|---|---|
| Flagella serotype[a] | Crystal serotype[b] | Epithet | | Curing/transfer | Hybridization | |
| 1 | thu | *thuringiensis (mattes) (berliner)* | 10 (5–150)<br>17 (3.9–180); 7 (5.4–58) | 75 | Chromosome, 42,55 | 29, 30, 103 |
| 2 | | *finitimus* | 77, 98 | 98, chromosome | 98 | 46a |
| 3a | ale | *alesti* | 12 (2–105)<br>6 (4–120)<br>10 (2.6–44.6) | 105 | <br>~120 | 29, 30<br>103<br>164 |
| 3a,b | k-1 | *kurstaki* | 12 (1.9–120)<br>8 (1.5–54) | 44,110 | 44, ~120 | 29, 30, 103<br>115 |
| 3a,b | k-73 | *kurstaki* | 6 (4.9–50) | 50 | 50 | 29, 30, 103; S. Minnich (unpublished data) |
| 4a,b | | *sotto* | 3 (5.2–43) | | 33–38 | 103 |
| 4a,b | | *dendrolimus* | 4 (33–73) | | Chromosome | 97 |
| 4a,c | | *kenyae* | | | | |
| 5a,b | gal | *galleriae* | 4 (5–130)<br>5 (6.3–74)<br>3 (5–160) | 130 | <br>~160 | 29, 30<br>115<br>103 |
| 6 | | *entomocidus* | | | Chromosome[c] | 116 |
| 6 | | *subtoxicus* | 2 (52,56) | | Chromosome and plasmid | 97, 115 |
| 7 | | *aizawa* | 12 (4–80)<br>8 (3.9–60) | | <br>45 | 90<br>98a, 115 |
| 8a,b | | *morrisoni* | 5 (10–160) | | 160 | 103 |
| 9 | | *tolworthi* | 9 (5–150)<br>6 (5.2–45) | | 45, 50, 150 | 103<br>115 |
| 10 | | *darmstadiensis* 73-G-10-2[d] | 4 (43–90) | | 47 | 138 |
| 11a,b | | *toumanoffi*<br>*kyushuensis*[d]<br>*wuhenensis* | 5 (10–160)<br><br>4 (5–150) | | 150<br>Chromosome | 103<br>136<br>103 |
| 12 | | *thompsoni* | 4 (4–100) | 100 | | 29, 30 |
| 14 | | *israelensis*[d] | 6 (4–72)<br>9 (3.3–135) | 75 | <br>75 | 74, 155<br>175, 176 |

[a] References 44 and 45.
[b] Reference 52.
[c] Lack of plasmids implies chromosomal location.
[d] Toxic only for Diptera; all with similar array of parasporal inclusions (as in Fig. 1) and polypeptides (Tam and Fitz-James, personal communication).

and *B. sphaericus* for control of mosquito and blackfly larvae is the requirement for formulations which keep the spores or inclusions or both in the feeding zone of the target pest in aquatic habitats (41, 94). In some cases, this feeding zone is just below the water surface; in others, suspension at a greater depth is necessary. In general, settling of spores or inclusions (or both) to the bottom of streams and ponds must be avoided.

## PROPERTIES OF *B. THURINGIENSIS* SUBSPECIES

There are literally thousands of isolates of parasporal body bacilli. Some of the more commonly studied subspecies are listed in Table 2. de Barjac and colleagues have provided some order on the basis of flagella serotypes (44, 45). Krywienczyk et al. (105–107) have extended this typing to include parasporal antigens. In general, they found a correlation between flagella (H) serotype and crystal serotype. For example, 38 of 56 *B. thuringiensis* subsp. *thuringiensis* (serotype H-1) isolates were of the thu crystal type; 8 of 9 *B. thuringiensis* subsp. *alesti* isolates were of the ale type, and 61 of 65 *B. thuringiensis* subsp. *galleriae* isolates were the gal crystal type (52). The exception to this predominance of one crystal type in each serological group was found among *B. thuringiensis* subsp. *kurstaki* isolates where there was a major division between k-1 and k-73 crystal types (105).

These types had very different activity spectra for larvae of *Trichoplusia ni* and *Heliothis virescens*.

Serologically identical crystals sometimes appeared in different H serotypes; e.g., k-1 crystals were present in both *kurstaki* and *thuringiensis* subspecies. Furthermore, some isolates contained a mixed crystal type, indicating that either one isolate may contain two protoxin genes or a given protoxin may share both major antigenic determinants.

The k-1 crystal type is particularly interesting since it was a *kurstaki* strain of this type which was originally found to contain two distinct toxins, the major lepidopteran protoxin (δ-endotoxin or P1) and a minor toxin (P2) active on both Lepidoptera and Diptera. k-1 crystals are also present in other subspecies and it is likely that both P1 and P2 will be found in these non-*kurstaki* isolates. Indeed, mosquitocidal isolates from subspecies *thuringiensis* and *kenyae* (as well as one from *tolworthi*) contained both P1 and P2 (182). Presumably these isolates were of the k-1 or mixed thu-plus-k-1 crystal type and thus fit with the correlation among k-1 type, mosquitocidal activity, and P1/P2 presence.

While mosquitocidal activity is often correlated with the k-1 crystal, it may not be restricted to this type. Two of three aizawai-type preparations were found to be mosquitocidal, although it was not determined whether P1 and P2 were present (52). In addition, there are at least three subspecies (*israelensis*, *kyushuensis*, and 73-G-10-2) all in different serotypes that are toxic only for mosquitoes. The morphology of the inclusions and array of parasporal proteins among the three are very similar (A. Tam and P. Fitz-James, personal communication) and clearly different from k-1 types (Fig. 1).

Crystal types have been further subdivided on the basis of activity spectra. Dulmage (52) examined the activity spectra of 38 isolates containing thu-type crystals and 36 k-1 types. They used activity ratios relative to a standard (HD-1-S-1971) for the larvae of two insect species to compare the relative potency of the toxins. The larvae compared were the following: (i) *Trichoplusia ni/Heliothis virescens*; (ii) *Hyphantria cunea/Bombyx mori*; (iii) *Hyphantria cunea/Trichoplusia ni*; (iv) *Bombyx mori/Heliothis virescens*; and (v) *Ephestia cautella/Plodia interpunctella*. The 38 thu preparations could be divided into 13 activity groups, the largest consisting of 10 isolates. Similarly, the 36 k-1 preparations were divided into 19 groups, the largest having four isolates. These subgroupings emphasize once again the complexity and variability of the *B. thuringiensis* crystals in that two isolates of the same crystal serotype may differ significantly in activity spectra. A possible explanation is that there has been exchange of plasmids (or protoxin genes or both) among these natural isolates. As will be discussed in greater detail, there is transfer of protoxin-encoding plasmids by cell mating (30, 71, 73, 129), and strains producing two different parasporal antigens have been constructed (71, 96, 129). Such exchange may occur naturally and contribute to new types via the presence of two or more different protoxin genes or even recombination between two such genes to create a new species of protoxin.

## CHARACTERIZATION OF PROTOXINS AND INCLUSION BODIES

A paracrystalline inclusion comprised of 130- to 140-kilodalton (kDa) polypeptides (δ-endotoxin or P1) is the predominant parasporal component of most *B. thuringiensis* subspecies. There are variations, however, in the number, shape, and composition of these inclusions (Fig. 1; Table 3).

### Comparative Properties

Of the crystal types listed in Table 3, the most prevalent is comprised primarily of one species of polypeptide of ca. 135 kDa (28, 34, 87). Extensive data on the morphology and biochemistry of this crystalline inclusion have been published (18, 28, 61, 86, 122, 124, 136, 137, 171), but for comparative purposes we note here some of its characteristics. Toxicity of these proteinaceous crystals for lepidopteran larvae was established by Angus in 1954 (5). Of the 14 *B. thuringiensis* flagella (H) serotypes, all but 1 (H-14) contain the P1 type of crystalline inclusion (122). There is usually one inclusion per cell, but there may be two or more (9, 14). Shape is characteristically bipyramidal (Fig. 1), but occasional irregular shapes have been observed (78, 86). Scherrer et al. (149) found that growth of *B. thuringiensis* in media containing >0.8% glucose resulted in amorphous crystals.

In most cases the crystal is found outside of the exosporium, major exceptions being subspecies *finitimus*, *B. cereus* subsp. *fowler* and *lewin*, and *B. popilliae* (9). It has been suggested that the exosporium and crystal may be closely associated (160, 162) but others have found that crystal formation did not necessarily occur close to the exosporium (9, 14), nor did it appear to involve mesosomes, forespore septa, or the forespore membrane (14).

Of the subspecies forming an inclusion within the exosporium, *B. thuringiensis* subsp. *finitimus* has been most extensively studied (46a, 157). This subspecies is not toxic for the larvae of Lepidoptera commonly tested, i.e., *Manduca sexta* or *T. ni*. The only significant toxicity reported has been for larvae of the cotton bollworm *Pectinophora gossypiella* (1). Recently, subspecies *finitimus* was found to produce both attached (within the exosporium) and unattached (outside the exosporium) inclusions each containing predominant 135-kDa polypeptides with unique immunological properties (46a). Plasmid-curing experiments indicated that the gene(s) necessary for formation of the larger, attached inclusion was encoded on a 98-megadalton (MDa) plasmid, while the gene(s) encoding the unattached body was chromosomally located. When the 98-MDa plasmid was transferred to *B. cereus* by cell mating, the transcipient produced an inclusion within the exosporium; i.e., some property of this large plasmid seemed to be involved in the site of deposition (or location) of the inclusion. The mechanism for enclosing the inclusion is not understood. There is a marked thinning of the exosporium in the region of the inclusion that may be part of this process.

A distinct class of inclusions is found in *B. thuringiensis* subsp. *israelensis* (Fig. 1). There are usually two to four inclusions per cell which vary in shape from cuboidal to bipyramidal, ovoid, or amorphous (32, 127, 185). They are relatively small (0.1 to 0.5 μm), and the toxic component is active against dipteran but not lepidopteran larvae (70). There is a broad spectrum of polypeptides extractable from these inclusions including a major 26- to 28-kDa protein that appears to be the toxin (7, 10, 185), although a 65-kDa species has recently been implicated (88, 114). The complexity of both the inclusions and polypeptides is reduced in partially plasmid-cured variants (Tam and Fitz-James, personal communication). The availability of such strains should permit a detailed analysis of the composition and function of the various inclusions.

Inclusions other than those containing known toxins have been observed in some strains of bacilli. These include ovoid inclusions in *B. thuringiensis* (14) and both ovoid and ellip-

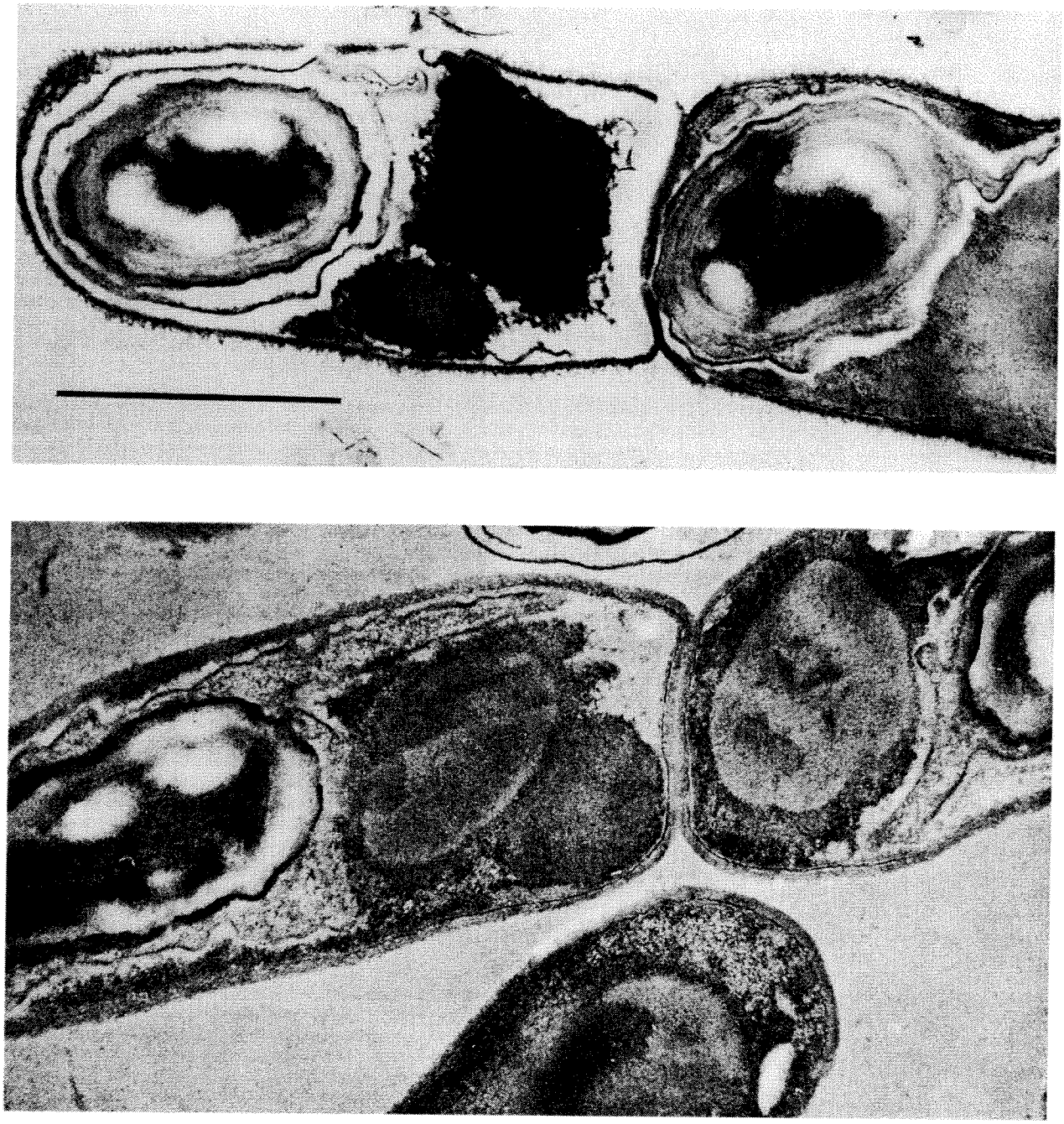

FIG. 1. Thin-section electron micrographs of two inclusion formers at stage VII of sporulation: (top) *B. thuringiensis* subsp. *kurstaki* showing the spore and ovoid and bipyramidal inclusions. Marker, 1 μm. (Bottom) *B. thuringiensis* subsp. *israelensis* showing the ripening, multicrystalline inclusions and, as in (a), typical *B. cereus*-type spores. ×47,000.

tical inclusions in *B. sphaericus* (39). There was no correlation between the presence of either of the *B. sphaericus* minor inclusions and toxicity (39). Their chemistry and function are unknown.

### Biochemical and Immunological Differences

Protein solubilized from inclusions of *B. thuringiensis* subsp. *israelensis* did not react immunologically with antibody to the *B. thuringiensis* subsp. *kurstaki* HD1 δ-endotoxin (106), and the overall amino acid compositions differ (171). In addition, there was no hybridization of a probe derived from the cloned *kurstaki* HD1 or HD73 protoxin genes with either total or plasmid DNA from subspecies *israelensis* (A. Aronson and W. Beckman, unpublished results). The δ-endotoxin appears to be a dimer of 230 kDa as shown by X-ray diffraction (85) and gel electrophoresis (87, 137). In sodium dodecyl sulfate gels the pre-

TABLE 3. Morphology of crystalline inclusions of selected bacilli

| Strains | Flagella serotype (H-type) | Crystals | | | | Reference(s) |
|---|---|---|---|---|---|---|
| | | Shape | Approximate length (μm) | Site of deposition | Time of formation | |
| *B. thuringiensis* subsp. *kurstaki*, P1 (δ-endotoxin) | 3a,3b | Bipyramidal | 1–1.5 | Outside exosporium | Stage II–III of sporulation | 9, 18, 86, 102 |
| *kurstaki*, P2 (mosquitocidal factor) | 3a,3b | Cuboid | 0.1–0.4 | Outside exosporium; often embedded in bipyramidal crystal | Just before P1 | 89 |
| *israelensis* | 14 | Cuboid, bipyramidal, ovoid, or amorphous | 0.1–0.5 | Outside exosporium | Stage II–III of sporulation | 172 |
| *finitimus* | 2 | Bipyramidal | 1–1.5 | Within exosporium | Stage II–III of sporulation | 9 |
| Other bacilli | | | | | | |
| *B. sphaericus* | | Polyhedron or no crystal | | Outside exosporium | | 39 |
| *B. medusa* | | | | Outside exosporium | At the end of vegetative growth | 9 |

dominant component is 130 to 140 kDa (33, 87, 171). In the case of *B. thuringiensis* subsp. *israelensis*, the major polypeptide in polyacrylamide gels (whether under native or denaturing conditions) is about 28 kDa (7, 171). Tyrell et al. noted that this species could result from proteolysis of a minor 134-kDa band (171). Subspecies *israelensis* preparations contain cytolytic and hemolytic activities (35, 109, 167) apparently in the mosquitocidal fraction (7, 176). More recently, *israelensis* parasporal crystal extracts were resolved into mosquitocidal and cytolytic fractions, with the former activity present in a 65-kDa protein (88, 114).

Differences between P1 and P2 are summarized in Table 4. Both appear to be protoxins (184), although only a small fragment of about 3 kDa is removed from P2. These two toxins are almost certainly encoded by distinct genes probably residing in different plasmids. The major *kurstaki* HD1 P1 gene is in a 44-MDa plasmid, perhaps with another gene in a larger plasmid (30, 75, 103). The location of the gene for P2 has not been definitively established. Loss of a 110-MDa plasmid from a *kurstaki* derivative, HD1-7, after growth at 42°C was accompanied by loss of P2 production (B. Carlton and T. Yamamoto, personal communication; A. Aronson, unpublished results). The resulting strain, HD1-9, also became conditional for P1 protoxin synthesis (129). The 110-MDa plasmid appears to have regulatory functions so its loss could indirectly affect P2 synthesis; i.e., the structural gene for P2 may be located elsewhere.

The mosquitocidal toxins of *B. thuringiensis* subsp. *kurstaki* and *israelensis* and of *B. sphaericus* differ by a number of criteria. The molecular weight of the *kurstaki* P2 is 65,000 (186), while for *israelensis* values of 26,000 to 28,000 or perhaps 65,000 have been reported (88, 171). The *B. sphaericus* toxin has been estimated at 55 kDa (169), but in an experiment with *E. coli* maxicells a DNA fragment containing a toxin gene(s) encoded four polypeptides of 21, 19, 15, and 12 kDa (120). It is not known whether one or all

TABLE 4. Differences between P1 and P2 of subspecies *kurstaki*

| Parameter | P1 | P2 | Reference |
|---|---|---|---|
| Toxicity | Lepidoptera | Lepidoptera and Diptera | 186 |
| mol wt (SDS-PAGE)[a] mol wt of protoxin | 135,000<br>55,000–70,000 | 65,000<br>62,000 | 186 |
| Shape of inclusion body | Bipyramidal | Cuboidal | 89 |
| Solubility | pH 10–12 + reducing agents | pH 10–12 | 184 |
| Time of synthesis | | Begins before P1 | 89 |
| Percentage of total crystalline inclusion protein | 70–90 | 10–30 | 89 |
| Isoelectric point | 4.4 | 10.7 | 186 |
| Chromatography (Sephacryl S-300) | Eluted before P2 | | 89 |
| Serology (rocket immunoelectrophoresis) | mobility toward (+) | mobility toward (−) | 183 |
| Tryptic peptide mapping | Complex pattern with resistant core | Relatively resistant | 182 |

[a] SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

of these are needed for toxin production, but a multipartite toxin, such as that produced by *B. anthracis* (173), is a possibility.

The *israelensis* toxin has been further differentiated from the *kurstaki* P2 toxin and the *B. sphaericus* toxin. Antiserum against *kurstaki* HD1 P2 crystals did not cross-react with the crystal protein of *israelensis* (184), nor did antibodies against total parasporal proteins from either *israelensis* or *kurstaki* HD1 cross-react (172, 180). A DNA probe containing the *B. sphaericus* toxin gene(s) hybridized specifically to DNA from toxic *B. sphaericus* isolates, but did not hybridize to *B. thuringiensis* subsp. *israelensis* DNA (120). *B. sphaericus* and subspecies *israelensis* have been tested in the field and found to be equally effective against the encephalitus vectors *Culex quinquefasciatus* and *C. annulirostris* and the potential malaria vector *Anopholes annulipes* (40). Their toxicity spectra were not identical, however, since *B. thuringiensis* subsp. *israelensis* was more effective against *Aedes* spp. and was also effective against blackfly larvae (40). The effectiveness of the *kurstaki* P2 protoxin relative to *B. sphaericus* and *B. thuringiensis* subsp. *israelensis* toxins has not been measured.

### Active and Nonactive Domains of the δ-Endotoxin

P1 is solubilized in the midgut of susceptible insect larvae and converted by gut proteases to a toxic moiety (6, 36, 113). Estimates of the size of the active toxin vary from below 5 kDa (62) to 80 kDa (86). Based on studies of crystal preparations digested with larval gut juice, Huber and Lüthy (86) concluded that the molecular weights of active polypeptides could vary from 80,000 to 30,000, with rapid loss of activity in fragments smaller than 30,000. More recently, Yamamoto and Iizuka (184) found that the protoxins of *B. thuringiensis* subsp. *kurstaki* HD1 and HD73 were digested by gut juice proteinases to a 62-kDa "resistant core" which retained full toxicity for test larvae. In other studies, toxic polypeptides of about 70 kDa were recovered from tryptic digests of crystal proteins from H serotypes 1, 2, 3, and 9 (118), and a toxic polypeptide of about 68 kDa was obtained after nonspecific degradation (due to endogenous proteases) at alkaline pH of solubilized *kurstaki* HD1 protoxin (20).

The more recent studies are in general agreement and are probably more accurate assessments of the size of the toxic moiety. It is important to measure specific toxicity and, if possible, the percent recovery of total toxicity. These determinations were not made in earlier work and could account for the broad range in size of toxic fractions. In addition, there may be some variation among subspecies, but a tentative range for the size of the active P1 toxin is 55 to 70 kDa.

The active portion of the P1 protoxin is located in the amino-terminal half of the polypeptide. Nagamatsu et al. (135) recovered a 58-kDa toxic fragment (T-fragment) from tryptic digests of the *B. thuringiensis* subsp. *dendrolimus* crystal protein and a similar sized toxic fragment was recovered from subspecies *kurstaki* HD263 (H. Arvidson and J. Aronson, personal communication). The amino-terminal amino acid sequence of the T-fragment was Ile-Glu-X-Gly-Tyr-Thr. This sequence matched that of the *kurstaki* HD1 (Dipel) gene (181) and of other genes as deduced from the nucleotide sequence starting at amino acid residue 29 (Fig. 2B). (HD1-Dipel is a commercially available derivative of *kurstaki* HD1. It is treated separately because its plasmid profile differs from that of HD1 [103].) Chestukhina et al. (33) also indicated that the amino-terminal portion of protoxins was retained in the toxin. They found amino-terminal methionine in both the protoxin and the stable fragment left after digestion with trypsin, a result not consistent with the experiments discussed above.

A presumptive carboxyl-terminal sequence of the toxin (Leu-Arg) is found at residues 499/500 or 510/511 of the Dipel gene. In either case a somewhat smaller toxic peptide than found by Nagamatsu et al. (135) would be produced. The difference may be due to comparing the gene structure from one subspecies with the toxic fragment from another and may be resolved with refinements in the analysis of the toxin. The T-fragment had a specific toxicity similar to that of the solubilized crystal protein, indicating that the first 28 amino acids and much of the carboxyl half of the protein were not essential for toxicity.

In the case of the *kurstaki* HD1 (Dipel) protoxin, location of the active domain at the amino-terminal half has been confirmed by deletion analysis (153). A series of deleted derivatives of the cloned gene was constructed, and a fragment that encoded an amino-terminal polypeptide of somewhere between 603 and 645 amino acids retained toxicity.

A subclone of the *kurstaki* HD73 protoxin gene that embraced the first 611 amino acids retained full toxicity (50% lethal concentrations) for *M. sexta* larvae, whereas a subclone encoding the first 596 amino acids produced no toxin in *E. coli* (W. Barnes and C.-L. Hu, unpublished results). These subclones encode polypeptides that are probably larger than the trypsin core toxin, so a portion of the gene product downstream from the core may be essential for proper processing of the toxic moiety. A similar situation may exist for the HD1 subclones (153).

The function of the carboxyl half of the protoxin molecule is not known although there appears to be considerable conservation at the amino acid level, at least for the four genes sequenced to date (Fig. 3). Surprisingly, the high-pressure liquid chromatography profiles of tryptic peptide digests of P1 from *kurstaki* HD73 and HD1 differed significantly (184). Since the toxic portion is resistant to trypsin, most of the peptides should have been derived from the carboxyl half, although the interpretation of the profiles may be complicated because of partial digestion. In addition, there is some evidence that restriction enzyme maps of this portion of protoxin genes from different isolates are more variable than the amino-terminal halves (98a).

Despite these differences, a direct comparison of the nucleotide sequences of several protoxin genes (Fig. 3) indicates that there is extensive conservation of the amino acid sequence of the carboxy half of these protoxins. There are a number of third-position differences and even use of alternate codons for a given amino acid. These differences may account for the variation in restriction enzyme maps but it is unlikely that these minor changes in the digested portion of the protoxin are critical for either toxicity or specificity.

Specific toxicity data of Nagamatsu et al. (135) and of Yamamoto and Iizuka (184) support this conclusion. In addition, the latter authors found that the activities of the protease-derived toxins from *kurstaki* HD1 and HD73 P1 protoxins against cabbage looper (*T. ni*) and tobacco budworm (*H. virescens*) larvae were virtually the same as the intact protoxins. These two *kurstaki* strains have different 50% lethal concentrations for these particular Lepidoptera (184) and since the relative activity was not altered in activated P1's, the carboxyl-terminal regions probably played no role in specificity. Unfortunately, the "acti-

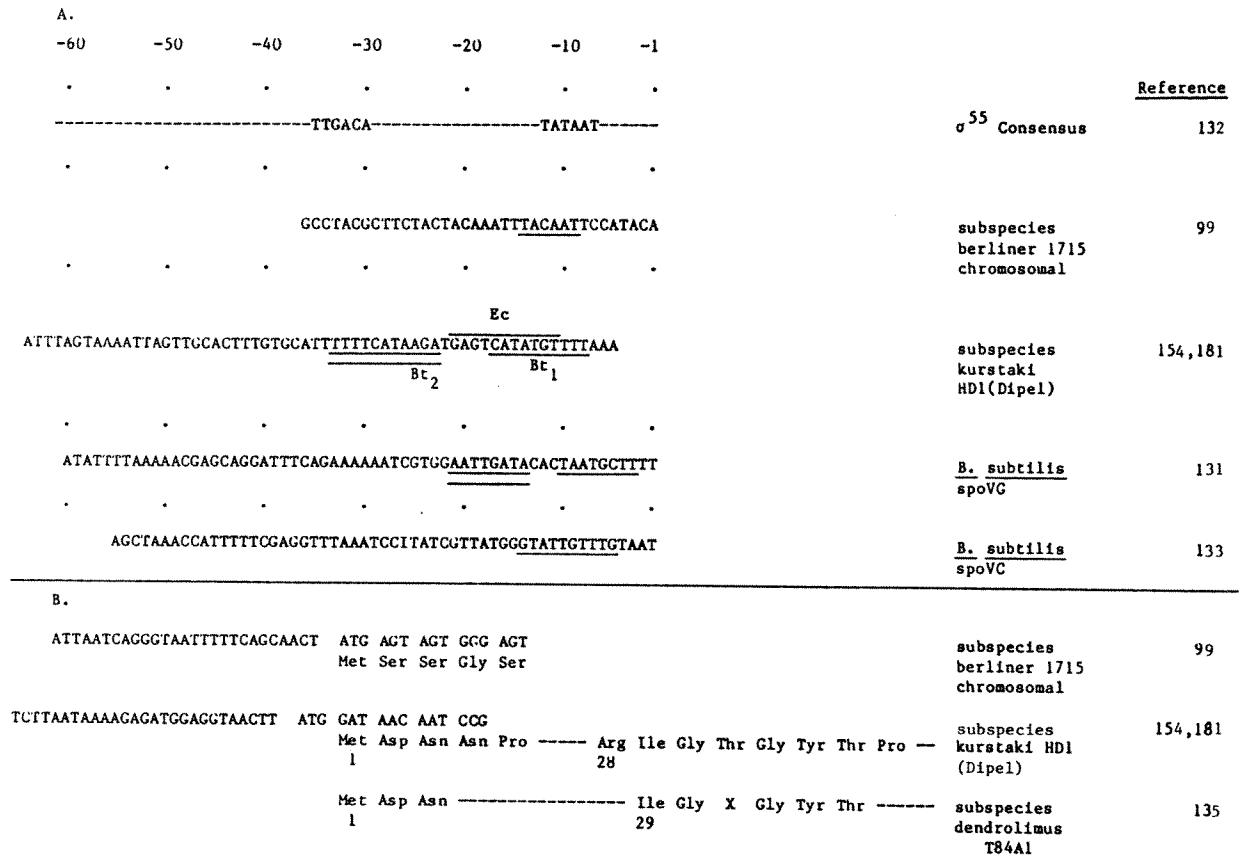


FIG. 2. (A) Comparison of sequences of promoter regions for two *B. thuringiensis* protoxin genes and two *B. subtilis* sporulation genes (*spoVG* and *spoVC*). The −10 regions are underlined. Double underlining indicates a second −10 region for those genes containing tandem promoters. The −10 region of the *B. thuringiensis* subsp. *berliner* 1715 gene was deduced from the sequence, while the others were established by S1 nuclease mapping. (B) Nucleotide and deduced amino acid sequences for the cloned *B. thuringiensis* subsp. *kurstaki* HD1 (Dipel) protoxin gene and for the subsp. *berliner* 1715 chromosomal "gene." The amino-terminal amino acid sequence of the protoxin and "activated" toxin (starting at residue 29) of *B. thuringiensis* subsp. *dendrolimus* is also included.

vated" protoxins and 135-kDa protoxins were not assayed in the same set of experiments.

In an earlier study (187), however, it was found that a proteolytic digest of crystals of subspecies *berliner* 1715 was toxic to *Anagasta* (= *Ephestia*) *kuehniella* larvae, whereas the intact crystal had relatively little effect, implying that either solubilization or removal of the carboxyl-terminal portion by proteases may enhance or broaden specificity. Thus the question remains whether the carboxyl-terminal half of the protoxin has some function in determining specificity. Other possible functions for this portion of the molecule are to protect the toxin (in the bacterium, soil, etc.), act in the attachment to gut epithelial cell receptors (63, 100), or act in the deposition of the protoxin as an inclusion. Cloned genes and subclones should be most useful for further defining the functions of various regions of protoxin molecules.

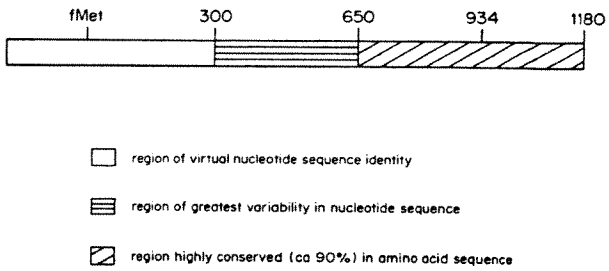


FIG. 3. Diagramatic comparison of nucleotide regions of four protoxin genes. Numbers above bar diagram indicate amino acid residues starting with fMet. Diagram based on data for clones from *B. thuringiensis* subsp. *kurstaki* HD1 (Dipel) (154), *sotto* (156), *kurstaki* HD73 (2; Barnes, personal communication), and *kurstaki* HD1 (Geiser, personal communication).

## CLONING AND GENE STRUCTURE

Toxin genes have been cloned from *B. sphaericus* and several subspecies of *B. thuringiensis*, including *israelensis* (Table 5). Clones of toxin genes from *B. anthracis* have been included for comparative purposes. These clones produced some toxin in *E. coli* (about 1 to 10% of an equivalent number of the bacilli from which the clone was derived), with two exceptions. The chromosomal *kurstaki* HD1 gene was originally detected by production of antigen and its toxicity for *M. sexta* larvae (83). However, a 4.6-kilobase (kb) *Eco*RI subclone was not reproducibly toxic nor did it

hybridize to a DNA fragment containing a plasmid-derived *kurstaki* HD1 protoxin gene or to the protoxin-encoding plasmid of subspecies *kurstaki* HD73, *berliner*, or *galleriae* (W. Beckman, and A. Aronson, unpublished data). Apparently, the *kurstaki* HD1 chromosomal clone contains a minor toxin which is not closely related to the major δ-endotoxin.

The chromosomal gene from subspecies *berliner* 1715 did not produce toxin detectable by immunological procedures or bioassay. This clone was used as a hybridization probe, however, for the isolation of a plasmid gene copy that did produce toxin in *E. coli* and *B. subtilis* so there must be at least partial sequence homology to an active gene. In fact, a 114-base pair (bp) sequence of this chromosomal gene is present in an HD1 gene (154). This chromosomal gene was not transcribed in *E. coli*; in *B. subtilis* it was transcribed (only in sporulating cells) but not translated (97). In addition, transfer of this clone to an acrystalliferous derivative of *kurstaki* HD1 by cell mating resulted in integration of the plasmid but no protoxin synthesis. In contrast, a clone of the plasmid-encoded *berliner* 1715 protoxin was stably maintained and produced protoxin when transferred to this *kurstaki* strain (97).

TABLE 5. Cloned toxin genes

| Strain | Gene location (MDa)[a] | Clone detection[b] | Reference(s) |
|---|---|---|---|
| *B. thuringiensis* subsp. | | | |
| *kurstaki* HD1 Dipel[c] | P (150) | 3 | 98a, 152; Geiser (unpublished data); Beckman and Aronson (unpublished data) |
| *kurstaki* HD1 | P (44) | 1, 2, 3 | |
| *kurstaki* HD1 | C | 1, 2 | 2, 83; Minnich and Aronson (unpublished data) |
| *kurstaki* HD73 | P (50) | 2, 3 | |
| *berliner* 1715 | P (42) | 3 | 97 |
| | C | 3 | 97 |
| *thuringiensis* HD2 | P (55) | 3 | 178a |
| *sotto* | P (33) | 3 | 98a, 156, 178 |
| *aizawa* | P (44) | 3 | 98a |
| *subtoxicus* | P (56) | 3 | 98a |
| *israelensis* | P (75) | 1, 2 | 155, 176 |
| *B. sphaericus* | C | 1 | 67 |
| *B. anthracis* | | | |
| PA | P (110) | 2 | 171 |
| LF | | | S. H. Leppla (personal communication) |
| EF | | | S. H. Leppla (personal communication) |

[a] Plasmid (P) or chromosomal (C).

[b] Recombinant clones were detected by: (1) toxicity to insect larvae; (2) reaction with toxin-specific antibody; or (3) hybridization to a gene-specific probe.

[c] The Dipel strain was isolated from a commercial preparation containing subspecies *kurstaki*. It is treated separately because its plasmid profile differs from that of HD1.

### δ-Endotoxin Genes

The promoter regions of five of the cloned δ-endotoxin genes have been sequenced: the protoxin genes of *B. thuringiensis* subsp. *kurstaki* HD1 (Dipel) (180) and HD73 (2; W. Barnes, unpublished results); a plasmid-encoded gene from subspecies *sotto* (156); another plasmid-encoded gene from subspecies *kurstaki* HD1 (M. Geiser, personal communication), and the chromosomal gene from *B. thuringiensis* subsp. *berliner* 1715 (97, 99). In Fig. 2A, only one sequence is shown since four of the five genes are virtually identical for several hundred nucleotides 5′ to the coding region and for the first ca. 900 bp in the coding region (Fig. 3). The *berliner* 1715 chromosomal gene differs considerably from the others but, as previously discussed, this gene is only transcribed and not translated in sporulating *B. subtilis* and produced no toxin when introduced into an acrystalliferous derivative of subspecies *kurstaki* HD1.

The active *kurstaki* HD1 (Dipel) gene was located by Tn*5* insertions and transcription initiation sites were determined by S1 nuclease mapping (181). There are three different "promoter" sites (Fig. 2A), one recognized by *E. coli* and two by *B. thuringiensis* RNA polymerases. Transcripts mapping to the downstream *Bacillus* promoter were found at about stage II of sporulation, but decreased at about midsporulation (stage III to IV), at which time transcripts from the upstream promoter appeared. The "−10 regions" for these promoters have no significant homology to the *B. subtilis* vegetative, $\sigma^{43}$ (the $\sigma^{55}$ factor has been found to be 43 kDa [69]), consensus sequence but they are related to promoter regions of certain *B. subtilis* sporulation genes. The downstream promoter −10 region has homology with the *spoVG* (131) (also characterized by two promoters) downstream −10 regions and also with the *spoVC* −10 region (133). A further similarity between the *B. thuringiensis* and *spoVG* genes is an adenine-thymine-rich region located about 50 bp upstream from the initiation sites. In *spoVG*, this adenine-thymine-rich region is required for transcription from the downstream promoter (131). Both *spoVG* and *spoVC* are activated early in sporulation and are recognized by an altered form of RNA polymerase containing a unique 37-kDa sigma factor ($\sigma^{37}$; 131, 133). The −10 region of the upstream promoter may have some homology to the "0.3-kb" gene of *B. subtilis* (139, 179, 181) which is transcribed beginning at stage III or IV of sporulation, possibly by a polymerase containing a unique sigma factor.

Similar altered polymerases have been found in sporulating *B. thuringiensis* (98). For example, it is known that certain *B. thuringiensis* δ-endotoxin genes can be transcribed by a unique sporulation polymerase referred to as the form II enzyme, but it has not yet been determined which promoters this enzyme recognizes.

The "−35 regions" of the two *kurstaki* HD1 promoters have no apparent homology to those from genes in *E. coli* or *B. subtilis* (132, 147; Fig. 2A). Lack of well-defined −35 regions is not a unique feature of δ-endotoxin promoters since the *spoVG* gene of *B. subtilis* also lacks a well-defined −35 region.

Another feature of the DNA sequence of the upstream region of the *kurstaki* HD1 gene is the presence of two regions of hyphenated dyad symmetry. These are located such that they include both transcription start sites and therefore could be sites where regulatory elements bind. Finally, the tandem promoters may contribute to regulation since they could ensure continued transcription by two distinct, altered polymerases present at different stages of

sporulation. The structure of the −10 regions suggests that a $\sigma^{37}$-like polymerase may transcribe early in sporulation from the downstream promoter, while a different form of RNA polymerase may transcribe from the upstream promoter beginning at midsporulation.

The translation start site was established by comparing the DNA sequence with the amino acid sequence at the amino terminus of the protein (181; Fig. 2B). A potential ribosome-binding site is present three bases upstream from the initiator ATG codon. The sequence has high complementarity (9 of 11 bp) to the 3′ end of the *B. subtilis* 16S ribosomal RNA and probably to *B. thuringiensis* 16S ribosomal RNA.

Transcription studies have been done with only one of the *kurstaki* HD1 genes (181), but it is likely that similar promoters exist for the four genes given the identical nucleotide sequences in the regulatory regions. Despite the identities of the *kurstaki* HD1 and HD73 genes, these toxins have different 50% lethal concentrations for certain Lepidoptera (184), and the plasmids containing these protoxin genes are regulated differently when transferred to *B. cereus* (129). Clearly factors other than promoter sequences and unique forms of RNA polymerase are involved in regulation.

### Chromosomal Protoxin Genes

Chromosomal protoxin gene sequences seem to be present in some subspecies (Table 2). There is preliminary evidence from Southern hybridization with a probe derived from a *B. thuringiensis* subsp. *kurstaki* HD1 cloned protoxin gene that protoxin genes are exclusively chromosomal in *B. thuringiensis* subspecies *dendrolimus* (97; but see reference 103) and subsp. *wuhanensis* (103). Hybridization per se does not establish gene copy number, whether or not the gene is functional, or rule out the possibility of plasmid-encoded protoxin genes unrelated to the particular probe used. As discussed previously, *B. thuringiensis* subsp. *finitimus* contains a chromosomal gene encoding a 135-kDa parasporal protein that is unrelated to any others (46a). The protein is immunologically distinct from the other major 135-kDa parasporal protein present in inclusions which are enclosed within the exosporium. In addition, DNA probes from the *B. thuringiensis* subsp. *kurstaki* HD1 or HD73 cloned genes reacted with a DNA fragment from a *Bam*HI digest of the 98-MDa plasmid but not with chromosomal DNA. The gene for the chromosomally encoded inclusion is apparently sufficiently different so that no cross-hybridization occurred even under conditions permitting 35% mismatch. The chromosomally encoded parasporal protein is synthesized later in sporulation than the plasmid-encoded protein, so gene location may reflect different modes of regulation.

As previously discussed, there is a cryptic chromosomal "gene" in *berliner* 1715 that has sequence homology to an active plasmid gene as well as to the *kurstaki* HD1 gene (97). In *kurstaki* HD1, a fragment of the chromosomal DNA cloned into a Charon phage was toxic for *M. sexta* larvae (83) even though plasmid-cured derivatives of this subspecies (still containing this chromosomal toxin gene) were nontoxic; i.e., the gene is apparently cryptic, at least in the absence of plasmids.

## REGULATION

Parasporal protein synthesis begins at about stage II or III of sporulation; i.e., completion of the forespore septum (14, 66, 112, 181) and the inclusion reaches maximum size which can approximate the size of the spore (135) by stage V. One exception to date is *B. medusa* in which inclusion formation begins at the end of exponential growth, prior to the formation of the forespore septum and often at a site distal to the eventual position of this septum (9).

The resulting bipyramidal crystal accounts for 20 to 30% of the total protein of the sporangium (112). In general, the time of appearance of the inclusion correlates with the increase in parasporal antigen and toxicity (3, 4) as well as of messenger RNA hybridizing with a cloned probe derived from the *B. thuringiensis* subsp. *kurstaki* HD1 protoxin gene (181). Activation of transcription and translation is thus closely coupled to protoxin accumulation.

The amount of protoxin produced must represent a substantial fraction of the protein-synthetic activity in these sporulating cultures. While there have been no direct measurements of the actual value, approximations can be made. The synthesis of protoxin antigen in *kurstaki* HD1 as detected by quantitative rocket immunoelectrophoresis occurred over about a 2-h period during sporulation to about 190 μg of antigen per ml (3) or about the amount of protein present in $3 \times 10^8$ cells. The number of cells in these experiments was not reported but is probably about $7 \times 10^8$ to $9 \times 10^8$/ml. The initial rate of protein synthesis in these sporulating cells is also not known but is certainly less than in exponentially growing cells (mean doubling time about 50 min). So it could take 2 to 3 h for cells to double their protein content, about the time required to accumulate 190 μg of protoxin antigen. On the basis of these assumptions, the rate of protoxin synthesis would be about 33 to 43% of the overall rate of protein synthesis.

In other assays, Andrews et al. (4) found that toxin antigen represented about 25% of the total alkali-soluble protein in a preparation of Dipel, a commercial mixture of spores plus inclusions. While alkali should dissolve all of the inclusion protein (18, 20), only the spore coat protein (30 to 40% of total spore protein) would have been soluble in alkali. Assuming no other source of protein, the inclusions should contain about 8 to 10% of the total protein in the Dipel. Here again, the time required to accumulate spore protein is not known but probably occurred over the 5 to 6 h necessary to complete sporulation. If so and if the protoxin accumulated in about 2 h as previously found (3), then the relative rate of synthesis of protoxin would be about 20 to 30% of the total rate of protein synthesis.

In most subspecies, the major protoxin gene is on a large plasmid (Table 2), and even if these were present in low copy number (i.e., less than five per cell as seems likely) with only one gene copy per plasmid, there could still be a considerable gene dosage effect. In organisms with more than one inclusion or perhaps more than one species of protoxin in a given inclusion, the contribution of gene dosage may vary.

There are additional bits of evidence that regulation probably involves factors other than gene dosage, unique promoter sequences, and sigma subunits. (i) Not surprisingly, expression of cloned protoxin genes in *E. coli* was poor (1% of the parental strains), but even in sporulating *B. subtilis* the clone of the *berliner* 1715 plasmid-encoded protoxin gene produced only about 10% as much protoxin antigen as the parental strain. (ii) Plasmid curing and transfer experiments indicated a role for several *kurstaki* HD1 cryptic plasmids in regulation (see next section). (iii) A *kurstaki* HD1 derivative (HD1-9) produced protoxin only at 25°C but sporulated well at both 25 and 32°C (129). This conditional phenotype could be suppressed, implicating cellular metabolic processes in regulation (see below). (iv) Cloned protoxin genes from

subspecies *kurstaki* HD1 and HD73 have identical nucleotide sequences both for the initial part of the coding region and for several hundred base pairs upstream, i.e., through the regulatory region (Fig. 3). Yet, when protoxin-encoding plasmids from HD1 or HD73 were transferred to *B. cereus*, only the latter expressed well, i.e., 30 to 50% of the parental strain. The 44-MDa plasmid from *kurstaki* HD1 produced little protoxin in *B. cereus* unless another HD1 plasmid of 4.9 MDa was present (129).

Similar results were found with a *B. cereus* transcipient containing the 98-MDa protoxin-encoding plasmid from subspecies *finitimus*. There were small inclusions (but enclosed within the exosporium) and much less 135-kDa parasporal antigen than in an equivalent number of parental cells. In this case, the plasmid-encoded protoxin gene has not been sequenced. The very different expression of protoxin-encoding plasmids in *B. cereus* transcipients, even when the nucleotide sequences of the regulatory regions were identical, indicates that other factors, probably plasmid encoded, are involved.

Conditional synthesis of protoxin in certain derivatives of subspecies *kurstaki* HD1 implied that regulation of protoxin synthesis may involve unique physiological factors that are not required for sporulation. It has been known for some time that variations in media influenced the relative yields of spores or inclusions (49). The conditional strain, HD1-9, sporulated well at either 25 or 32°C but produced protoxin only at the former temperature (129). This regulation probably occurred at the level of transcription since there was no detectable protoxin messenger RNA in sporulating cultures of HD1-9 grown at 32°C. Conditional synthesis also was found in *B. cereus* transcipients containing the 29-MDa plus 44-MDa, or the 29-, 44-, and 4.9-MDa plasmids from *kurstaki* HD1. As discussed in the next section, the 29-MDa plasmid (in the absence of one of 110 MDa) seemed to be responsible for the conditional phenotype.

The conditional phenotype of strain HD1-9 could be specifically suppressed by a subinhibitory concentration of D-cycloserine or in a D-cycloserine-resistant derivative of HD1-9 (8). Since neither the conditional phenotype nor its suppression affected sporulation, there must be regulatory signals specific for protoxin synthesis.

None of the studies have addressed the regulation of the time of expression of protoxin genes. In all of the *B. cereus* transcipients and in one case for the cloned protoxin gene from *berliner* 1715 in *B. subtilis* (97), expression was confined to postexponential (sporulating) cells. As discussed above, the promoter regions seem to be unique and probably require special forms of RNA polymerase that are functional only in sporulating cells. The temperature-sensitive factor involved in the conditional *kurstaki* strain HD1-9 appeared to be synthesized during exponential growth (129), but here again protoxin synthesis at the permissive temperature occurred only in sporulating cells.

In *B. subtilis*, the presence of unique forms of RNA polymerase is necessary but not sufficient for the expression of sporulation genes. Several but not all of the unique sigma factors seem to be present prior to the time they are functional and to require products of at least some of the Spo0 loci. For example, Zuber and Losick (189) found that fusions of the promoter for the *spoVG* gene to β-galactosidase were not transcribed in most *B. subtilis* Spo0 mutants. These Spo0 mutations are pleiotropic, affecting the function not only of some $\sigma^{29}$ and $\sigma^{37}$ promoters but also of $\sigma^{28}$ promoters (68). The regulatory action of the Spo0 gene products may or may not be direct, but clearly other components are involved in regulating the transcription of these sporulation genes.

## FUNCTIONS OF *B. THURINGIENSIS* PLASMIDS

In general, *B. thuringiensis* subspecies contain a substantial portion of their potential genetic information in plasmids (Table 2). The number and sizes of the plasmids vary considerably. In subspecies with a larger number such as *berliner* 1715 or *kurstaki* HD1, there is a very broad range in size, often with a cluster around 4 to 6 MDa and then several larger than 30 MDa, with some greater than 100 MDa. In subspecies with only a few plasmids such as *subtoxicus* or *finitimus*, they tend to be larger.

There is some variation in plasmid profiles of the same subspecies in data from different laboratories. These variations probably reflect the following. (i) Different procedures were used for lysing cells resulting in variation, in particular, in the recovery of large plasmids (>100 MDa). It appears that the protoplast lysis procedure of Eckhardt (59) as modified by Gonzalez and Carlton (72) is best suited for displaying the entire array of plasmids. (ii) Differences may also arise because of variations in plasmid profiles in separate isolates of the same subspecies (103). As will be discussed, there is evidence for IS sequences, especially in large plasmids, and many of these embrace protoxin genes (104, 117). The transfer of plasmids via cell mating may also be important. Both factors may contribute to variations in plasmid profiles for different isolates with the same flagella serotype or for a given strain kept under laboratory conditions for a prolonged period.

### Regulation

In *B. thuringiensis* subsp. *kurstaki* HD1, there is evidence that certain plasmids that do not contain protoxin genes have a role in regulating the synthesis of protoxin (129). A derivative that was isolated after several steps of growth at 42 and 30°C had lost several plasmids (5 of 12) but still produced parasporal inclusions. Loss of a 110-MDa plasmid from this strain (HD1-7) by growth at 42°C resulted in strain HD1-9 that now produced protoxin only when grown at 25°C but not at 30 to 32°C. Assuming no other modification than loss of a 110-MDa plasmid, the conditional phenotype had apparently been suppressed by some function of this large plasmid. A second change in HD1-9 was the inability to produce the P2 toxin.

Conditional protoxin synthesis was also obtained by transfer of protoxin-encoding plasmids from subspecies *kurstaki* HD1 to *B. cereus* 569 (129). Plasmid transfer from *kurstaki* HD1 occurred at a low frequency (<1%) compared with subspecies *kurstaki* HD73 (30 to 40%; 71), but as discussed in the next section, prototrophs were selected and screened for plasmid content. In all cases, a plasmid of 29 MDa was transferred; some also had plasmids of 44 MDa (containing the major protoxin gene) and one of two had plasmids of 4 to 6 MDa. These three to four plasmids among the 12 in *kurstaki* HD1 were transferred predominantly. *B. cereus* transcipients that had gained the 44-MDa protoxin-encoding plasmid now produced protoxin but only when grown at 25°C. In addition, the amount of protoxin produced increased 5- to 10-fold if a plasmid of 4.9 MDa were also transferred. Thus, the small, cryptic plasmid seemed to have a function in regulating the amount of protoxin produced, at least in *B. cereus* transcipients.

The conditional phenotype of the transcipients was apparently due to the 29-MDa plasmid since transfer of a 50-MDa

protoxin-encoding plasmid from subspecies *kurstaki* HD73 into *B. cereus* transcipients containing the 29-MDa plasmid from *kurstaki* HD1 resulted in protoxin synthesis only when cells were grown at 25°C. In contrast, transfer of this 50-MDa plasmid to *B. cereus* not containing the 29-MDa plasmid resulted in substantial protoxin synthesis in cells grown at either 25 or 30°C. At least in *kurstaki* HD1, cryptic plasmids of 110, 29, and 4.9 MDa have functions in regulating protoxin synthesis. It is intriguing that the plasmids most frequently transferred to *B. cereus* in cell matings were those with a regulatory function in protoxin synthesis (29 and 4.9 MDa) plus one containing a major protoxin gene (44 MDa).

This complex regulatory pattern does not seem to apply to the closely related subspecies, *kurstaki* HD73, since transfer of the 50-MDa plasmid to *B. cereus* seems to be sufficient for protoxin synthesis at both 25 and 30°C and in amounts approaching (30 to 50%) the parental strain. Subspecies *kurstaki* HD73 has a simpler plasmid profile (Table 2) so that regulatory genes may have been incorporated into the same plasmid that carries the protoxin gene.

In contrast, transfer of the 98-MDa protoxin-encoding plasmid from subspecies *finitimus* to *B. cereus* resulted in the formation of small inclusions enclosed within the exosporium (as in the parental strain). These transcipients produced much less 135-kDa parasporal protein (either protein stain or antigen in immunoblots) than the donor strain. Presumably there are regulatory factors encoded by the other finitimus plasmid (77 MDa) or by the chromosome which are not present in the *B. cereus* transcipients. Inclusions were found only in *B. cereus* transcipients grown at 25°C. In this case, however, growth at 30°C resulted in loss of the 98-MDa plasmid from transcipients (in contrast to the parental strain or *B. cereus* transcipients containing *kurstaki* HD1 plasmids), so some chromosomal function of *finitimus* may be involved in plasmid maintenance.

It will be necessary to elucidate the regulatory functions of the *kurstaki* HD1 cryptic plasmids to determine whether or not similar functions exist in other subspecies. Subspecies *kurstaki* HD1 may be unique in the complexity of its regulatory genes (reflecting the multiple number of protoxin genes) or perhaps in their location.

### Protoxin Genes

Most *B. thuringiensis* plasmids are cryptic but a few functions have been assigned, including bacteriocin production (Aronson, unpublished results) and protoxin synthesis (103). Initially, protoxin synthetic capacity was correlated with the presence of plasmids in complete (42, 161) or partial (72, 75) curing studies and later via plasmid transfer in cell mating experiments (Table 2). Following the cloning of protoxin genes from *B. thuringiensis* subsp. *berliner* 1715 and *kurstaki* HD1 (97, 152), probes were available for examining other subspecies for the presence of related sequences, presumably intact protoxin genes. On the basis of cross-hybridization, protoxin genes were found primarily in large plasmids and, in some cases, on more than one plasmid in a given subspecies (97, 103, 152). There is also evidence for related or unrelated chromosomal protoxin genes as discussed earlier.

Protoxin genes seem to be in plasmids of various sizes, but in all cases the plasmids are >30 MDa. The variety of locations may be related to the presence of inverted repeat elements in the vicinity of protoxin genes (104, 117), possibly providing a mobilizing capacity. In addition, Gonzalez and Carlton (74) have evidence for the mobility of a protoxin gene from a study of the plasmid profiles and toxicity of *B. thuringiensis* subsp. *israelensis* derivatives. Some spontaneous derivatives had lost the protoxin-encoding plasmid of 75 MDa but sequences hybridizing with this plasmid were present in the chromosomal DNA. These derivatives were still toxic for mosquito larvae so the toxin gene from the 75-MDa plasmid had presumably integrated in such a way that expression was still possible. Derivatives of this strain were found which were still toxic but now contained 75-MDa plasmid sequences in new plasmids of 65 and 80 MDa. Among the 75-MDa plasmid sequences in these new plasmids were presumably those encoding the toxin since loss of the 65- and 80-MDa plasmids resulted in nontoxic derivatives containing only a portion of the 75-MDa plasmid sequences in the chromosome. These results have recently been confirmed by using a clone of the *israelensis* toxin gene as a probe (155). The simplest explanation for the occurrence of these variants is the presence of the *israelensis* "protoxin" gene on a transposable element. The presumptive movement into and out of the chromosome may serve as a model to account for the presence of protoxin sequences in the chromosome of some subspecies.

### Cross-Homologies and IS-Like Sequences

Southern hybridization has revealed some homology among the plasmids isolated from different serotypes (115). In general, the two sets of plasmid probes used (isolated from either *B. thuringiensis* subsp. *dendrolimus* [at least 33 MDa] or *B. thuringiensis* subsp. *subtoxicus* [52 and 56 MDa]) did not react with smaller plasmids from any of the other nine subspecies tested but there was some cross-hybridization among plasmids larger than 30 MDa. Conversely, small plasmids (<9 MDa) from *B. thuringiensis* subsp. *berliner* 1715 hybridized exclusively with small plasmids from the two other subspecies tested, *B. thuringiensis* subsp. *kurstaki* HD1 and *israelensis*. The 4.9-MDa plasmid of *kurstaki* HD1 was isolated from a strain cured of all other plasmids by growth in a subinhibitory concentration of mitomycin C. This plasmid hybridized only with plasmids of similar size in several other subspecies (Aronson and Beckman, unpublished results). This subdivision according to size may reflect a common origin and perhaps function(s) of these plasmids. The absence of small plasmids (and related sequences) in some subspecies, however, implies that their function(s) is not essential for growth or parasporal inclusion formation, although a few essential sequences may have been integrated into larger plasmids.

One unique plasmid sequence appears to be common among several large plasmids, especially those containing protoxin genes (116). A general class of sequences with inverted repeats was originally noted by Dababov et al. (43). This particular sequence, apparently an IS-like element, was found as an insertion in a *Streptococcus faecalis* plasmid introduced into *B. thuringiensis* (116). Its presence was then noted by Southern hybridization in several large plasmids, particularly in regions close to the protoxin gene of subspecies *berliner* 1715 (117).

A more direct approach was used to isolate sequences with inverted repeats from subspecies *kurstaki* HD73 (104). Snap-back sequences were isolated following S1 nuclease digestion of fragments reannealed under conditions where intramolecular base pairing could occur. Here again, these sequences were found primarily in large plasmids, including those containing protoxin genes. In the case of *kurstaki*

HD73, several such sequences were localized to a region within 1 to 3 kb of the protoxin gene. It is unlikely that these IS-like elements from the two subspecies are identical since their distribution patterns differ. Sequencing of both classes will provide definitive data on their relatedness.

Other functions encoded by *B. thuringiensis* plasmids must be more subtle since strains of subspecies *kurstaki* HD1 essentially lacking all plasmids have been isolated after heating spores (163) or growing cells at 42°C. These strains were originally reported to lack all plasmids but many contain a 130-MDa plasmid of unknown origin not present in the parental strain (J. Gonzalez and B. Carlton, personal communication). These derivatives do not produce bacteriocin or parasporal inclusion proteins and are thus lacking two major plasmid-associated functions and probably many others. They have no major alteration in growth requirements and grow and sporulate about as well as the parental strain in complex or minimal media. Many of these "plasmid-free" isolates produced spores deficient in spore coat (10), a phenotype anticipated since the parental strains form coat-deficient spores coated with parasporal protein (see section on spore coat synthesis). The absence of this protein in these plasmid-free derivatives accentuated the spore coat deficiency.

As indicated, the overall growth requirements and growth rates of these plasmid-free derivatives were essentially unchanged. In several cases, the growth yield in complex media was less by 10 to 20%, implying functions for the plasmids during late or postexponential growth. In fact, a general analysis of plasmid transcription patterns of *kurstaki* HD1 by Northern hybridization (Aronson, unpublished results) indicated that many major transcripts were found primarily in sporulating cells. It is thus likely that one of the functions of these plasmids is to enhance or provide supplementary growth factors when nutrients become limiting. For example, these plasmid-free derivatives sporulated poorly when resuspended in a salts-glutamate medium lacking phosphate, in contrast to the parental strain. One of the cryptic plasmid functions may be to mobilize phosphate (via permease, phosphatase, etc.) for starved cells.

A detailed analysis of sporulation requirements of *B. thuringiensis* may reveal other functions for these plasmids. Perhaps a better assessment of in vivo conditions may be necessary to fully appreciate the role of these diverse plasmids in the physiology of these organisms.

## GENETICS OF *B. THURINGIENSIS*

### Mapping by Transduction

Several *B. thuringiensis* generalized transducing phages have been isolated and used for preliminary mapping studies (11, 81, 108, 110, 142, 168). While different subspecies have been used, some preliminary linkage data have emerged (Table 6). Despite the use of different transducing phages and subspecies, there appears to be uniformity in the order of the markers. The antibiotic resistance group ordered by Landén et al. (108) and Heierson et al. (81) appears to overlap with linkage group III of Lecadet et al. (110). Similarly, their group III contains markers present in group III of Barsomian et al. (11). Group I in *B. thuringiensis* subsp. *berliner* 1715 seems to overlap with group I of *B. thuringiensis* subsp. *aizawai*; group II in subspecies *berliner* 1715 containing the *metC(D)* and *argO* genes is likely to be part of linkage group II defined in subspecies *aizawai*. The only unique linkage group to date is group IV in *berliner* 1715, but more data are obviously necessary to fill in the gaps.

TABLE 6. Transduction mapping in various *B. thuringiensis* subspecies

| Transducing phage | Subspecific epithet | Linkage group | Reference |
|---|---|---|---|
| φ63 | *gelechiae* (serotype 1) | *nalA–nifA–strA–spcA* | 108 |
| φ64 | *gelechiae* | I: *guaB–purB–metA–novA(purA–nalA)–rifA–strA–spcA*<br>II: *hisA–lysA* | 81 |
| CP54Ber | *berliner* 1715 (serotype 1) | I: *asp–his-1–lys-1–cys-4*<br>II: *metC* or *-D argO*[a]<br>III: *metA–purA–cysA–rif*<br>IV: *gly-1–ura-1–gly-2* | 110 |
| TP13 and TP18 | *aizawai* (serotype 7) | I: *(trp-1 trp-2)–(leu-1 leu-2) his-1–(lys-1 lys2)–cys-1*<br>II: *met-1–(argC1 argO1)–met-2–(pyr-1 pyrA2)*<br>III: *met-3–pur-1 (nal-1 nal-2) str-1 (pur-2 pur-4)–pur-3* | 11 |

[a] Based on analogous phenotypes in *B. subtilis* (81).

There are some similarities to the *B. subtilis* chromosome (84): (i) the linkage of *metC(D)* and *argO*; (ii) the clustering of related biosynthetic markers such as those for *leu*, *trp*, *pyr*, and *pur*; (iii) the linkage of antibiotic resistance markers that affect transcription or translation; (iv) linkage of a purine marker (possibly *purA*) to nalidixic acid resistance; (v) linkage of *cysA* to rifamicin resistance (presumably *rpoB*); and (vi) the order of the markers in linkage group II of Barsomian et al. (11). Heierson et al. (81) have discussed similarities and differences in their linkage group from subspecies *gelechiae* (H serotype 1) with *B. subtilis*. Obviously, more data are needed, but the availability of transducing phage carrying various sized fragments of the *B. thuringiensis* chromosome (11) should be useful in ordering markers and in joining linkage groups.

### Plasmid Transformation

While there are no reports of transformation of *B. thuringiensis* cells, there is some evidence for plasmid transformation of protoplasts, albeit at a very low frequency (65, 125, 130). The procedures used were essentially those developed for *B. subtilis* (31), using high concentrations of lysozyme to convert cells to protoplasts. *B. cereus* (and presumably the closely related *B. thuringiensis*) cells contain primarily nonacetylated murein (80). The lack of *N*-acetyl groups results in lysozyme resistance so the protoplasts may form because of either activation of autolysins or perhaps the removal of a small amount of *N*-acetylated murein present in critical regions of the cell. Whatever the basis for the lysozyme conversion, these protoplasts are viable, i.e., increase in size in osmotically stable nutrient media but revert poorly if at all in a variety of soft-agar media (Aronson, unpublished results).

Protoplasts of *B. thuringiensis* form at a relatively low frequency (10 to 40%) if exponentially growing cells are resuspended in 0.3 M sucrose–0.01 M $MgCl_2$ buffered at either pH 7.4 or 6.5 and shaken slowly at 37°C for 60 to 90 min (Aronson, unpublished data). Presumably, certain autolysins become activated under these nongrowing conditions. These protoplasts may be transformed with plasmids via the polyethylene glycol procedure (31) and reversion occurs on soft agar containing the appropriate antibiotic. For example, we have transformed *B. thuringiensis* subsp. *kurstaki* HD1 with plasmid pBC16 (15) at an approximate frequency of one per $10^5$ to $10^6$ protoplasts. This *B. cereus* plasmid has been used for cloning in *B. megaterium* (174). It is stable in *B. thuringiensis* and may be readily transferred to other subspecies or to *B. cereus* via cell mating. In addition, it seems to have mobilization functions that allow transfer of other cryptic plasmids (A. Aronson, unpublished). Ruhfel et al. (148) were able to transduce pBC16 from *B. cereus* into various *B. thuringiensis* subspecies and other *B. cereus* strains. The plasmid could then be transferred along with other *B. thuringiensis* plasmids containing protoxin genes by cell mating (12).

### Cell Mating: Plasmid and Chromosome Transfer

Transfer of plasmids via cell mating of *B. thuringiensis* was first reported by Gonzalez et al. (71, 73). They found a high frequency of plasmid transfer, especially one of 50 MDa containing the protoxin gene from *B. thuringiensis* subsp. *kurstaki* HD73 to *B. cereus*, acrystalliferous derivatives of HD73, or *B. thuringiensis* subsp. *thuringiensis* HD2. Transfer was also found from a subspecies *kurstaki* strain (HD263-1) to HD73 and *B. cereus* and from a derivative of subspecies *thuringiensis* HD2 to *B. cereus*. In many cases, plasmids containing protoxin genes were transferred and the recipient cells (transcipients) produced protoxin with antigenic properties identical to the donor strain. In some matings, transcipients containing two distinct protoxin-encoding plasmids were constructed in successive steps. These transcipients produced both species of protoxin as demonstrated by using specific antibodies in an Ouchterlony imunodiffusion assay.

In all of these cases, the plasmids did not contain a selectable marker so the only selection was for recipient cells. These were screened at random for plasmid profile or production of an inclusion or both so the frequency of plasmid transfer in these cases had to be fairly high.

These cell matings were initially done by slowly shaking the cells. The plasmid transfer was deoxyribonuclease resistant and probably required cell contact. The number of subspecies that transferred plasmids at a high frequency was very limited, however. In some cases, such as subspecies *thuringiensis* HD2, transfer was found only when derivatives that had lost certain cryptic plasmids spontaneously or by curing were used as donors (71). For *B. thuringiensis* subsp. *israelensis* HD567, transfer of the protoxin-encoding plasmid was found only to a plasmid-free *israelensis* derivative and then at a low frequency (74). Other plasmids were transferred more frequently and one of ~135 MDa seemed to be essential for the transfer of the 75-MDa protoxin-encoding plasmid. It appears that in many subspecies other cryptic plasmids may interfere with plasmid transfer. One or more of these cryptic plasmids may code for a bacteriocin that could kill potential recipients. Others may alter the surface properties of donor cells and thus affect the formation of mating pairs.

There is one report of the transfer of cloned protoxin genes from *B. subtilis* to *B. thuringiensis* by cell mating. Klier et al. (96) found that a clone of the *berliner* 1715 plasmid-encoded, protoxin gene (pBT42-1) was efficiently expressed when introduced into an acrystalliferous, plasmid-free strain of subspecies *kurstaki* HD1. It is surprising that these distantly related species could mate, but it may provide a means for introducing DNA cloned in a *B. subtilis* vector back into *B. thuringiensis*.

The recombinant plasmid (pBT42-1) containing the plasmid-encoded active *berliner* 1715 δ-endotoxin gene was also transferred from *B. subtilis* to *B. thuringiensis* subsp. *israelensis*. Both the *berliner* 1715 (lepidopteran active) and *israelensis* (dipteran active) toxins were expressed in normal amounts (96). This result is significant for three reasons. (i) It demonstrates that the gene transfer system may be used to introduce cloned genes into *B. thuringiensis* strains already containing a protoxin-encoding plasmid, (presumably the recombinant plasmid needs *tra* functions or some other way to be mobilized in *B. subtilis*). (ii) Transfer of pBT42-1 into *B. thuringiensis* subsp. *israelensis* resulted in the expression of two different toxins in the same strain in confirmation of other mating experiments (71, 129). This compatibility could be of great practical importance for broadening the host range of strains used in biological control. (iii) The high level of expression of the recombinant plasmid's toxin gene in *B. thuringiensis* is in sharp contrast to the low levels of expression in *E. coli* and *B. subtilis*.

In an analogous experiment, Lereclus et al. (116) found that the *S. faecalis* plasmid pAMβ1 could be introduced into *B. thuringiensis* by filter mating. These strains could now serve as plasmid donors in mating experiments that use the selectable markers from pAMβ1 (lincomycin/erythromycin resistance) to enrich for transcipients containing this plasmid. In addition to transfer of pAMβ1, there was high frequency of transfer of other cryptic plasmids present in the donor strain.

So one procedure for improving the detection of plasmid-containing transcipients is to introduce a plasmid such as pBC16 or pAMβ1 carrying a selectable marker that is capable of mobilizing other cryptic plasmids. It is also possible to select for transfer of chromosomal markers by using appropriate auxotrophic recipients (129). Among the prototrophic transcipients, there was a very high frequency (~100%) of transfer of plasmids from the donor strain, including those containing protoxin genes. For example, plasmid transfer from subspecies *kurstaki* HD1 to *B. cereus* 569 is so low (<1%) as to be virtually undetectable by randomly picking *B. cereus* colonies. Transfer of chromosomal markers by cell mating on filters occurs at a frequency of about $1/10^7$ and all of the prototrophs contained at least one HD1 plasmid (129). If an HD1 derivative, HD1-9, were used as the donor, chromosomal marker transfer frequencies increased about 100-fold. HD1-9 was derived in several sequential plasmid-curing steps (growth at 42°C as well as random screening) from HD1 (B. Carlton and J. Gonzalez, personal communication). In particular, its immediate parent, HD1-7, contained a 110-MDa plasmid. Loss of this plasmid resulted in conditional synthesis of protoxin (129), loss of the ability to synthesize protoxin P2, and an increase in chromosomal marker transfer of about 20-fold (Aronson, unpublished results).

The frequency of marker transfer varied in the order *trp* > *met* > *his*. There may be sequential marker transfer with an order that is consistent with the order of the markers in transduction analysis. Obviously, more markers are needed

and the relation of these markers to those studied in other subspecies must be determined.

Chromosomal marker transfer occurred best in filter matings and was fairly resistant to deoxyribonuclease (40% reduction in the transfer of *trp*). The 29-MDa plasmid of *kurstaki* HD1, which was always found in the transcipients, appeared to be essential for chromosome mobilization since there was no marker transfer if *kurstaki* HD1 strains lacking the 29-MDa plasmid were used as donors or if HD1-9 derivatives with an altered 29-MDa plasmid were used. There is a ca. 2-kb region of homology between the 29-MDa plasmid and the chromosome of *kurstaki* HD1 (Beckman and Aronson, unpublished results) that may be involved in chromosome mobilization.

As discussed elsewhere, of the 12 or so *kurstaki* HD1 plasmids, 3 were predominantly found in *B. cereus* transcipients: the 29 MDa, one of 44 MDa that encodes a major protoxin gene (152), and one or two of 4 to 6 MDa that appear to be involved in regulating the amount of protoxin produced in these transcipients (129). Consistent with the data of Gonzalez et al. (29, 30, 71) was the frequent transfer of a protoxin-encoding plasmid.

By using a similar approach, the protoxin-encoding 98-MDa plasmid of subspecies *finitimus* was transferred to *B. cereus* auxotrophs by selecting for prototrophic transcipients. In this case, the donor strain was one cured of the other major *finitimus* plasmid of 77 MDa. Loss of this plasmid resulted in lack of bacteriocin production, a factor that may have enhanced survival of recipient *B. cereus* cells and thus an apparent increase in prototrophic recombinants. About 33% of the *B. cereus* prototrophs contained the 98-MDa plasmid and produced a small inclusion, largely within the exosporium, the site of a major inclusion in the parental strain. The other 67% may have lost the 98-MDa plasmid since its maintenance in *B. cereus* is temperature dependent. There was no detectable homology between the 98-MDa plasmid and *finitimus* chromosomal DNA so the mechanism of chromosome mobilization, if it occurs, may not be the same as in subspecies *kurstaki* HD1. The frequency of chromosomal marker transfer, however, was much lower with the *finitimus* derivative as a donor (Debro, unpublished results).

The capacity for genetic exchange of both plasmid and chromosomal markers among *B. thuringiensis* subspecies and with *B. cereus* could at least in part account for the variation of parasporal antigens within given flagella serotypes as discussed in an earlier section. Mixtures analogous to some of the isolates described by Dulmage (52) have been constructed via cell mating (75), in part reflecting the ready transfer of the 50-MDa protoxin-encoding plasmid of subspecies *kurstaki* HD73. Other mixtures involving subspecies *berliner* 1715 and *israelensis* (96) and *kurstaki* HD1 and *israelensis* (129) have been constructed so the capacity to mobilize certain plasmids, especially those containing protoxin genes, may contribute to the mixing of parasporal antigens within given flagella (H) serotypes.

It is also conceivable that there could be recombination among these plasmids or with chromosomal genes to further contribute to a greater heterogeneity of parasporal antigenic types. Recombination could occur between two parasporal protein genes leading to a hybrid species and thus provide the basis for the evolution of these related protoxins. The extensive nucleotide similarity of certain regions of protoxin genes (Fig. 3) could be a factor in such an exchange. Alternatively, recombination could lead to two protoxin species becoming stably integrated, perhaps accounting for the multiplicity of protoxins within subspecies such as *finitimus* and *kurstaki* HD1 or even for the presence of silent, cryptic genes as may be the case for the chromosomal genes in subspecies *berliner* 1715 (97) and *kurstaki* HD1 (83).

Much of this speculation is based on the finding of genetic exchange following the mixing of high numbers of cells in liquid or on filters. Soil microorganisms seldom reach sufficient concentrations where cell mating could be a major factor for genetic exchange unless there were pheromones produced as in the streptococci (57). Alternatively, cell concentrations within the hemolymph of insect larvae do get very high and would be ideal for cell mating, assuming a mixed population were present. Plasmid or chromosomal gene exchange may then occur among subspecies containing protoxins with overlapping specificities. As new isolates are found, it will be interesting to follow the patterns of heterogeneity in parasporal antigenic types with similar or identical flagella serotypes. These patterns may provide information about overlapping specificities of protoxins or the occurrence of various subspecies in given environments, i.e., where certain mixtures of insects may be prevalent.

## OTHER SPOREFORMING BACILLI

There are a number of apparently related bacilli capable of proliferating in the hemolymph of larvae from the beetle family *Scarabaeidae*. *B. popilliae* and *B. lentimorbus*, two of the most common isolates, are facultative anaerobes but only members of the former group produce parasporal inclusions. The relationship of this structure to pathogenicity is not known. Review articles written in the last several years (19, 95, 128) have summarized the practical concerns and problems in the use of this organism for the control of members of the family *Scarabaidae*, i.e., *Popillia japonica* (Japanese beetle) and related organisms. Advances in the biology of *B. popillae* have been very limited because of the great difficulty in producing pathogenic, sporulating cultures in the laboratory. While some isolates form a parasporal inclusion, this structure is not essential for toxicity (95), at least for *P. japonica*. It would be of interest to compare the *B. popilliae* inclusion protein (and perhaps the gene) and those produced by *B. thuringiensis* subspecies.

One similarity to the parasporal body-forming bacilli is the relationship of insect toxicity to sporulation. Toxicity is apparently associated with the spore structure (as is the δ-endotoxin of *B. thuringiensis*; see below) since the supernatant of sporulating cultures was nontoxic (95, 128). There have been reports of the apparent instability of toxic strains in soil (19, 95), suggesting that toxicity may be plasmid encoded like many of the *B. thuringiensis* protoxins (Table 2). Obviously, many questions and much speculation will be resolved when suitable laboratory cultivation conditions are found for producing pathogenic cultures in reasonable yields.

Similar problems for achieving good sporulation in culture exist for *Bacillus larvae*, a pathogen for honeybee larvae. *B. larvae* is also a facultative anaerobe (for growth at least) and is catalase negative, as are *B. popilliae* and related species. Dingman and Stahly (47, 48) have developed a liquid medium providing good sporulation of *B. larvae*. A proper nutrient environment and a low level of aeration were both essential.

*B. sphaericus* has been discussed to some extent in the section on characterization of protoxins. It is ubiquitous in soil and soil-aquatic systems, but strains of most interest have been isolated from dead mosquito larvae primarily of

*Culex* spp. and *Anopheles* spp. and to some extent *Aedes* spp. Spores or sporulating cultures of these isolates that were fed to larvae were toxic. Some isolates have broad toxicity among species of mosquitoes whereas most are specific for certain species (158). There is a correlation between the presence of a certain type of parasporal inclusion and toxicity (39, 46, 92), although there are toxic isolates that apparently lack inclusions (37). In these cases, toxic activity copurified with the cell wall/membrane fraction (37–39). Solubilization of toxin was achieved by treating spores plus inclusions with 0.05 N NaOH. This treatment solubilized the inclusion without disrupting the integrity of the spore but would not rule out some alkali-soluble toxin on the spore surface in analogy with *B. thuringiensis* (10). Since some Diptera, like the Lepidoptera, have alkaline guts, similar solubilization of a toxin in an inclusion or on the spore surface could occur. The alkali-extracted proteins appeared to have molecular weights of 35,000 to 54,000 on the basis of elution from Sephadex G100 and sodium dodecyl sulfate gel electrophoresis (38). A value of about 55 kDa was reported for the soluble toxin following ion-exchange chromatography and gel electrophoresis (169). Given the early conflicting reports on the size of the *B. thuringiensis* protoxin (reviewed in references 122, 137), this range for the *B. sphaericus* toxin must be considered tentative. Problems with the δ-protoxin stemmed largely from endogenous proteases that either functioned in cultures kept for prolonged periods or were not inactivated during cell lysis and solubilization. Indeed, in a recent report, Baumann et al. (13) provided evidence for a 43-kDa toxin that may be derived from a precursor of >100 kDa. Because it is difficult to be certain that proteolysis has been controlled, confirmation of the size of the toxin may depend on sequencing of the cloned gene(s) (67).

As previously discussed, *B. sphaericus* is only one of several isolates with mosquitocidal activity. Antibody to *B. sphaericus* toxic extract did not cross-react with *B. thuringiensis* subsp. *israelensis* toxin or vice versa (38). In addition, the *B. sphaericus* toxin gene(s) has been cloned on a 3.7-kb fragment (67) and very old *E. coli* cells containing this clone produced almost as much toxin as the parental strain. The selective expression of this gene in very old *E. coli* cultures is unusual. Perhaps there are unique transcription factors in *E. coli* that function only in nutrient-depleted (or oxygen-limited) conditions and coincidentally recognize the *B. sphaericus* toxin-gene promoter. The *B. sphaericus* fragment did not hybridize with subspecies *israelensis* DNA, again indicating very different toxins (120). Hybridization occurred primarily with chromosomal DNA from toxic *B. sphaericus* strains, perhaps another example of a parasporal inclusion protein encoded by a chromosomal gene(s). The comparative DNA sequences of the cloned *B. sphaericus* and subspecies *israelensis* toxin genes will be of great importance in attempting to understand the structure and mode of action of these very different mosquito toxins.

## OTHER CONSIDERATIONS

### Relation of Spore Coat Synthesis and Germination to Parasporal Body Formation

In comparison to *B. cereus*, the spores of most subspecies of *B. thuringiensis* germinate slowly (10). The tests involved heat activation and addition of germinants known to be effective for *B. cereus* such as L-alanine and inosine. Other germinants (dipicolinic acid, glucose, Penassay broth) have been tried with no effect. It is conceivable that the correct germinants specific for *B. thuringiensis* have not yet been found. These subspecies are very closely related to *B. cereus* in a number of ways, however, so it is likely that they have similar spore structures and germination triggers. In addition, the spores of many acrystalliferous, plasmid-cured isolates of subspecies *kurstaki* HD1 have complete coats as judged by electron micrographs and gel electrophoresis of spore extracts. These spores responded to L-alanine and inosine as well as did *B. cereus* spores.

For some reason, the germination mechanism is defective in spores produced by strains forming parasporal inclusions. There is a deficiency of spore coat in such strains, and this deficiency may include a germination trigger or perhaps the incorrect orientation or inaccessibility of the germination system.

In those subspecies with thinner spore coats, there was deposition of protoxin on the spore surface so the spores per se were toxic unless washed so as to remove the loosely bound protoxin. Most *B. thuringiensis* subspecies examined (*kurstaki* HD1 and HD73, *galleria*, *alesti*, *finitimus*) had protoxin on the spore surface and the spores germinated slowly. The one exception to date is *B. thuringiensis* subsp. *israelensis*, which produced relatively nontoxic, rapidly germinating spores (10). Electron micrographs and the pattern of spore coat-extractable proteins indicated complete *B. cereus*-like spore coats in subspecies *israelensis*. The parasporal proteins extractable from *israelensis* inclusions differ from those present in inclusions of most other *B. thuringiensis* subspecies and perhaps these small proteins do not bind readily to the spore surface.

Subspecies *israelensis* may be analogous to a *B. cereus* transcipient containing protoxin-encoding plasmids. These transcipients, in contrast to the donor strains (*kurstaki* HD1 or HD73), still form spores with complete spore coats and there is little, if any, deposition of protoxin on the spore surface. Apparently a second event must occur to coordinate protoxin synthesis with the decreased formation of spore coat protein.

There may be a functional relationship between the formation of coat-deficient spores and the deposition of protoxin on the spore surface. These spores are almost as toxic per microgram as the purified parasporal inclusion (10, 150, 171). If the main purpose of protoxin synthesis by these bacilli is to provide a unique nutrient-rich environment, i.e., the larval hemolymph, then it could be considered advantageous to produce spores containing protoxin (or inclusions within the exosporium as in the case of subspecies *finitimus*).

There is evidence in these bacilli for regulation of spore coat synthesis that is correlated with protoxin synthesis (Fig. 4). In some acrystalliferous subspecies *kurstaki* HD1 derivatives cured of plasmids by heating of spores, the spore coats were very thin, resulting in lysozyme sensitivity of the spores in some cases (18, 163). In most isolates, there was only a thin coating on the spores (less than that in the parental strain), implying that lack of protoxin may contribute to instability of the coat or inefficient deposition. Alternatively, the curing procedure, i.e., spore heating, may have resulted in other genetic alterations due to loss of other plasmids or some undefined mutation.

It is interesting, however, that it was possible to obtain lysozyme-resistant spores (about $1/10^6$) with full coats and *B. cereus* germination properties from one of these acrystalliferous mutants (Fig. 4). In addition, most plasmid-cured, acrystalliferous strains isolated after treatment with sublethal concentrations of mitomycin c produced spores

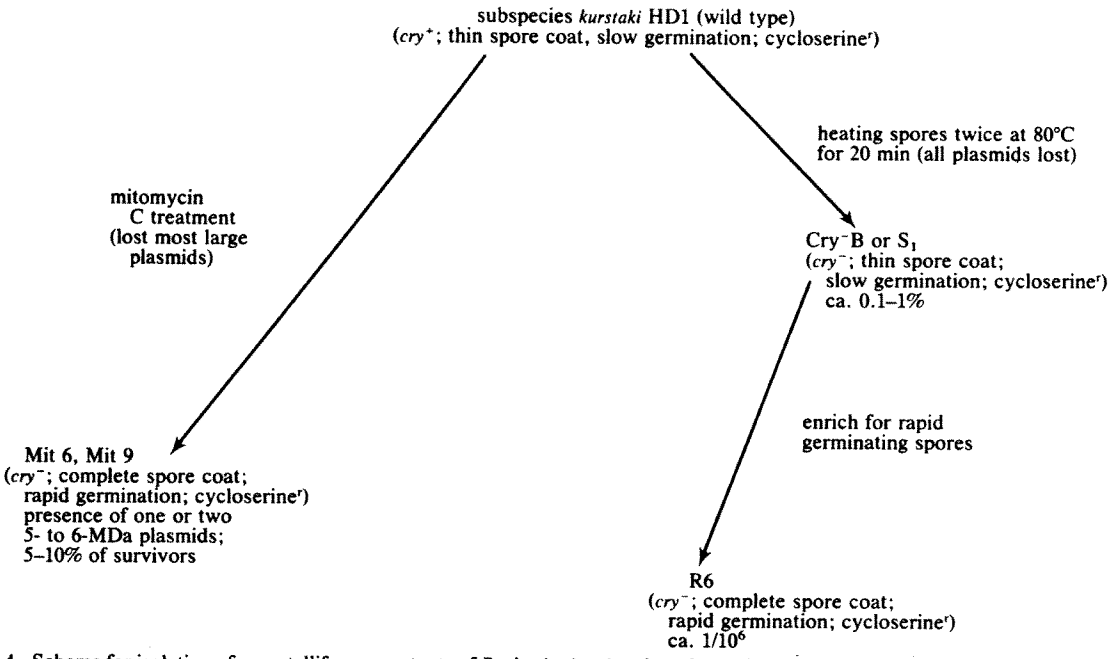


FIG. 4. Scheme for isolation of acrystalliferous mutants of *B. thuringiensis* subsp. *kurstaki* HD1 with or without complete spore coats. For germination studies see reference 10. Numbers given are approximate frequencies of occurrence of each type.

that germinated well and contained full spore coats. It appears that subspecies *kurstaki* has the capacity to produce "complete" spore coats, but this potential was not fully expressed probably due to some property of protoxin-encoding plasmids. As previously mentioned, the mere presence of these plasmids was not sufficient, however, since introduction of protoxin-encoding plasmids into *B. cereus* did not result in an alteration of spore coat synthesis and the spores germinated as rapidly as the parental *B. cereus*.

### Persistence

The rather sluggish germination of many *B. thuringiensis* subspecies may contribute to the lack of persistence of these organisms in the soil. Such a difference in germination rate in the soil could be a factor in a fairly rapid dilution of protoxin-producing species in the absence of larval hosts, although spores of *B. thuringiensis* subsp. *aizawai* were lost after 135 days from inoculated soil samples under conditions where the spores did not germinate (177). Another factor could be the loss of plasmids, especially those containing protoxin genes. In many subspecies, plasmids are lost by growth at 42°C and even at 37°C there may be loss. As discussed above, such plasmid-cured strains can regain a complete spore coat and a rapid germination response and would thus be virtually identical to *B. cereus*.

In some cases, the major criterion for persistence is killing of larvae so the lack of availability of spores or inclusions at sites of larval feeding would constitute a narrower but more relevant estimation of persistence. Both factors may be important in the long-term effectiveness of this organism. Survival in the soil would involve not only the rate of response to germinants but the availability of larval hosts, killing by bacteriocins, etc. Further study of the biological implications of a sluggish spore germination system would be of interest.

### Bioassays

Research efforts to improve formulations of these organisms, to increase the toxicity spectrum of various isolates, or to understand the mechanism of action of toxins produced by these bacilli rely heavily on bioassays against target pests. Several forms of bioassay of *B. thuringiensis* spores, crystals, or toxins against lepidopteran larvae have been developed. These include measurements of mortality (55) or decreased feeding (22, 121) by (i) larvae force-fed preparations (50), (ii) larvae fed leaves of plants that have been coated with solutions to be assayed by dipping (50) or spraying (27), or (iii) larvae fed artificial diet with incorporated materials (55) or surface treatments (151). The choice of test insect, life stage assayed, method of administering the test substance, parameter measured as an indicator of toxicity, and environmental conditions may all affect the results of the bioassay and, thus, must be carefully chosen (50). As an example, if the tobacco hornworm *M. sexta* is chosen as the test organism, little difference between surface treatment of diet and incorporation of samples was noted (148). However, considerable variation in results with these two methods of sample administration were noted in assays with the tobacco budworm *H. virescens*, an insect which both feeds on the surface and burrows into the diet (50).

An alternative to whole-animal bioassays is available for soluble preparations of toxin proteins. These assays rely on the sensitivity of certain insect cell lines, primarily the CF-1 line isolated by Sohi (159) from the spruce budworm *Choristoneura fumiferana*, to activated toxin from parasporal crystals (134). In these assays, the activity of toxin preparations may be measured by histopathological observation (134), by vital staining (167), by quantitating cell lysis

(166), by assaying cellular adenosine triphosphate levels (91), or by quantitation of the release of intracellular enzymes such as lactate dehydrogenase (58).

In 1971, a standardized assay utilizing larvae of the cabbage looper *T. ni* was adopted by U.S. industry and by the Environmental Protection Agency for official comparison of samples (55). This assay is standardized by comparison of potencies of tested materials to a primary international reference preparation of the HD-1 isolate (23, 51).

In bioassays of materials against aquatic dipteran larvae, an entirely different set of problems related to keeping the test material in the feeding zone of the test species is encountered (24). Further, some species require static and others require moving water to ensure ingestion of the test material (24). A standardized method for bioassay of *B. thuringiensis* subsp. *israelensis* against larvae of vector species of mosquito has been developed by the World Health Organization (mimeographed document TDR/ BCV/ BTH14/861WHO/VBC/81.828, 1981). Davidson (37) has described a bioassay of soluble toxin from *B. sphaericus* against larvae of the mosquito *Culex quinquefasciatus*.

### Mode of Action

Discussion of the mode of action of preparations of *B. thuringiensis* is complicated by the number of subspecies of the pathogen studied by different investigators, the number of susceptible hosts studied, the production of more than one toxic material by the bacterium, the possible interaction of spores and crystals in toxicity to some larvae, and evidence that the toxic materials may act at several sites in susceptible hosts. Among the many potentially toxic materials produced by *B. thuringiensis*, β-exotoxin and δ-endotoxin are generally regarded as the most significant in pathogenicity to insects. The β-exotoxin is an adenine nucleotide which inhibits RNA synthesis in insects and other animals (111). As a result of the toxicity of β-exotoxin to some vertebrates, however, it is excluded from all strains used for commercial production of insecticidal products sold in Western countries except Finland (24).

The δ-endotoxin is the major component of the characteristic parasporal crystals produced by these species. Heimpel and Angus (82) have classified lepidopteran larvae into three types based on their susceptibility to crystalline endotoxin, bacterial spores, or mixtures of the two. Type I insects are killed by preparations of crystalline δ-endotoxin alone and spores of the bacterium do not increase toxicity; type II insects are susceptible to endotoxin but the effect is enhanced by the presence of spores; and type III insects are only killed by spore-endotoxin mixtures. The midgut pH of most susceptible larvae is too alkaline to allow spore germination but is suitable for dissolution and activation of protoxin; thus most susceptible insects fall into type I. In some cases, midgut pH may be closer to neutrality, allowing germination, or the action of endotoxin may cause a decrease in pH so that germination can occur (24, 26, 36, 82). Most studies of the mode of action of *B. thuringiensis* have focused on type I insects where toxicity appears to be due to the action of the δ-endotoxin alone.

The first sign of poisoning following ingestion of crystalline endotoxin by type I insects is paralysis of the gut and mouth parts, leading to a cessation of feeding (36, 82). This sympton appears within minutes and its rapid onset, before dissolution and proteolytic activation of the protoxin, suggests that it may be induced by intact protoxin. Subsequent to the onset of gut paralysis, there is a swelling of the microvilli present on the lumenal surface of midgut epithelial cells, swelling of the cells themselves, changes in endoplasmic reticulum and mitochondria, disruption of ion and glucose transport and oxygen uptake, loss of adenosine triphosphate from midgut cells, and eventual separation of midgut cells from the basement membrane and bursting of separated cells in the midgut lumen (24, 36, 54, 123). Disruption of midgut structure and function leads to ion and pH imbalances in the hemolymph, total body paralysis, and death. Cooksey (36) has reported that toxin, but not unactivated protoxin, affects ion transport and function of neurons in isolated cockroach ganglia. However, the main site of action appears to be the midgut epithelium. Investigations of the mode of action of activated toxin on the midgut epithelium have suggested that it may act as an uncoupler of mitochondrial oxidative phosphorylation (170). An effect on the transport of $K^+$ ions by isolated midgut epithelium of *M. sexta* has been reported by Griego et al. (77). The studies of Harvey and Wolfersberger (79) have demonstrated that the toxin specifically inhibits an active electrogenic $K^+$ pump located in the plasma membrane of midgut epithelial cells and these authors concluded that effects on mitochondria were secondary.

Histopathological and biochemical studies of the toxicity of activated toxin on cultured insect cells (reviewed in reference 123) have provided evidence for a mode of action similar to that described above. There is some evidence that the toxin produced by *B. thuringiensis* subsp. *kurstaki* may act as a lectin binding to *N*-acetylgalactosamine residues exposed on the surface of cells (100). In similar studies with the 26-kDa cytolytic toxin produced by *B. thuringiensis* subsp. *israelensis*, however, there was binding to phosphatidylcholine, sphingomyelin, and phosphatidylethanolamine, indicating that this toxin may act as a detergent to disrupt membrane integrity (166). Such nonspecific detergent action is not consistent with the established species specificity of this toxin. It is possible, however, that specificity derives from the activation of protoxin due to unique conditions present in the midguts of mosquitoes and black flies.

## OUTSTANDING PROBLEMS

The development of genetic exchange systems for both plasmid and chromosomal genes and the expression of cloned toxin genes in *E. coli* have greatly aided progress on toxin gene structure and regulation. These approaches will be most useful for elucidating the basis of toxin action and specificity. Comparative structures of genes for toxins effective on dipteran larvae, i.e., those produced by *B. sphaericus*, subspecies *israelensis* (and perhaps *kyushuensis*), and *kurstaki* HD1 strains should help in determining whether these toxins overlap in structure and mode of action. Further comparisons of δ-endotoxin genes should be useful for establishing conserved regions and perhaps providing a framework for the origin of toxin diversity, i.e., independent origins or via recombination among these genes in strains that contain two or more protoxin-encoding plasmids.

Regulation of protoxin synthesis will certainly involve the unique promoter regions and altered forms of RNA polymerase in analogy to the *B. subtilis* sporulation polymerases (119). It is likely that factors unique to *B. thuringiensis* are also required so that regulation studies will eventually have to be done in the parental organism rather than with clones in *E. coli* or *B. subtilis*. For these purposes, reproducible procedures for plasmid transformation must be developed.

Further characterization of the genetic exchange systems will also be most helpful.

There is also the question of multiple protoxins (or parasporal proteins) as well as multiple genes in some subspecies. Some of these genes, especially certain chromosomal sequences, appear to be cryptic and may be pseudogenes or genes that function only in special conditions. In at least one case (subspecies *finitimus*), there are both functional chromosomal and plasmid genes, each encoding unique parasporal proteins. Gene location may simply reflect the transposition of a progenitor protoxin "unit" to various environments in different isolates or may be a manifestation of a more elaborate regulatory network involving the capacity of the cell to synthesize inclusion proteins in response to a variety of environmental or metabolic signals.

The expression of protoxin genes seems to be integrated with other metabolic events occurring in postexponential cells. The deposition of protoxin on the spore surface appears to be more than a coincidental affinity of this protein for the spore surface. Most *B. thuringiensis* subspecies examined produce spores which are deficient in coat and in germination response. These surface alterations may be related to the rather efficient deposition of parasporal protein on the spore surface. Since derivatives producing spores containing complete coats can be isolated, a regulatory system may exist that is integrated with protoxin synthesis.

Several isolates, especially those toxic for dipteran larvae, produce complex inclusions that contain an array of proteins. Since only one or a few of these polypeptides are toxins per se, the others may have ancillary roles in the process. If so, their synthesis would presumably be regulated with the toxins. In addition, there are reports of enhancement of toxicity by spores of the same subspecies as the inclusion (26). The function of the spores is not known but could involve specific protoxin activation, production of toxin "enhancers," or even other types of toxins that could complement those present in the inclusion. The possibility that regulation of toxin synthesis involves an array of protein and structural components must be further considered. These organisms may contain elaborate systems for enhancing infectivity or toxicity or both.

Despite the gaps, it is almost certain that protoxin synthesis and related processes are geared to providing these organisms with a unique ecological niche. Hemolymph provides an excellent nutrient environment for proliferation and thus the rare opportunity for soil microorganisms to reach concentrations sufficient for genetic exchange via cell mating. It seems likely that insect larvae from a number of groups could serve as hosts for specific soil microorganisms. The recent finding of a new *B. thuringiensis* subspecies toxic for coleopteran larvae (102) supports this hypothesis and suggests that many unique bacteria are yet to be found. The potential practical applications are obvious.

Among the practical considerations to be dealt with are persistence and the inevitable problem of larval resistance. Learning more about the regulation of protoxin synthesis and the number of factors involved should be helpful in appreciating why these organisms may or may not persist in a particular environment. Protoxin gene stability (plasmid replication properties, etc.), germination rates, nutritional requirements, etc., will all play a role. Persistence specifically at sites of larval feeding probably involves additional factors.

Widespread use of these organisms as in grain storage bins has already resulted in the appearance of resistant insects (126). More knowledge about specificity, protoxin conversion to toxin, attachment to larval cells, and the mode of action of various toxins will be helpful in understanding mechanisms of resistance. The capacity to engineer protoxin genes in clones should prove useful for overcoming at least some resistance problems and perhaps in designing an array of toxins that could be used in combination to decrease the frequency of appearance of resistant insect populations.

Excellent progress has been made in recent years on this intriguing system. Clearly further research in the basic aspects of protoxin synthesis and regulation will provide not only insights into a unique process of secondary metabolism, but also essential information for the practical applications of these microorganisms.

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# EXHIBIT F

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# Insecticidal Activity of *Bacillus laterosporus*

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Strains of *Bacillus laterosporus* demonstrated pathogenic activity for second-instar larvae of the mosquito, *Culex quinquefasciatus*, but failed to demonstrate detectable pathogenicity against larvae of the cabbage looper, *Trichoplusia ni*. Of 29 strains of the bacterium screened, 16 displayed pathogenicity for mosquito larvae. One of the most pathogenic strains, NRS 590, also demonstrated pathogenic activity for larvae of the mosquito, *Aedes aegypti*, and for larvae of the black fly, *Simulium vittatum*. The pathogenicity for *Culex* larvae was associated with the cell mass rather than with the culture supernatant. A suspension of ultraviolet irradiation-killed cells demonstrated no loss in pathogenic activity, an indication that the pathogenicity is toxin mediated. The toxic substance produced by NRS 590 was found to be resistant to heating at 96°C for 10 min. The toxin was not associated with the heat-resistant, bacterial endospore or with the associated paraspore since a suspension consisting primarily of spores was not toxic to mosquito larvae. Toxic activity in stationary phase cells of NRS 590 was associated with the cell's particulate fraction rather than with the soluble fraction. 

KEY WORDS: *Bacillus laterosporus; Culex quinquefasciatus*, larvae of; *Trichoplusia ni*, larvae of; *Simulium vittatum*, larvae of; insect pathogenicity; microbial insect pathogen.

## INTRODUCTION

*Bacillus laterosporus* is an aerobic, spore-forming bacterium which was originally isolated from water (Laubach, 1916; White, 1920). It is distinguished from similar bacilli by the production of a canoe-shaped parasporal body which cradles the spore and is firmly attached to it. The ultrastructure and chemistry of the spores and paraspores have been studied by Hannay (1957) and Fitz-James et al. (1958). The isolation of *B. laterosporus* from diseased bees (McCray, 1917) suggested that this bacterium might be an insect pathogen. However, *B. laterosporus* is recognized to be a saprophyte living on the dead remains of bee larvae and it is not always present in these insects (Bailey, 1981). A strain of *Bacillus* identified only as *B. laterosporus/alvei* was reported to be pathogenic for larvae of the cigarette beetle, *Lasioderma serricorne* (Singer, 1981). Because there has been so little information published regarding the possible role of *B. laterosporus* as an insect pathogen and because of the presence in the cell of a parasporal inclusion comparable to those found in pathogenic strains of *Bacillus thuringiensis* and *Bacillus sphaericus*, this bacterium was examined in detail. This paper reports the results of screening *B. laterosporus* strains for pathogenicity to larvae of the mosquito, cabbage looper, tobacco budworm, and black fly. It also reports the presence and some characteristics of toxic activity toward mosquito and black fly larvae.

## MATERIALS AND METHODS

*Bacterial growth conditions and culture media.* The strains of *B. laterosporus* employed in this study are listed in Table 1. *B. laterosporus* was grown in NYSM broth (8 g/liter Gibco nutrient broth supplemented with 0.5 g/liter Difco yeast extract, $7 \times 10^{-4}$ M $CaCl_2 \cdot 2H_2O$, $5 \times 10^{-5}$ M $MnCl_2 \cdot 4H_2O$ and $1 \times 10^{-3}$ M $MgCl_2 \cdot 6H_2O$). Flasks containing 10% of their volume as liquid were inoculated (5%, v/v) with bac-



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teria grown at 30°C overnight in static tubes of NYM broth (NYSM broth lacking the salts). The flasks were shaken (175 rpm) in a New Brunswick Model G25 shaker (New Brunswick Scientific, New Brunswick, N.J.) at 30°C. For growth cycle studies, an inoculum consisting of vegetative cells was prepared by transferring cells two times between shaken flasks (NYM broth) when the absorbance reached 0.20 (660 nm). The final flasks (200 ml of NYSM broth in a 2-liter flask) were then inoculated (10%, v/v) and shaken (175 rpm) at 30°C. Growth was monitored using a Klett–Summerson photoelectric colorimeter (red filter). For the 4-hr sample, 200 ml of culture was harvested by centrifugation, washed once in distilled water, and suspended in 15 ml of distilled water. For the remaining samples, 15 ml of the culture was harvested, washed once with distilled water, and suspended in 15 ml of distilled water. Total viable cell numbers were determined by plating on NYSM agar after diluting in sterile, distilled water. Spore numbers were determined by heating 2 ml of sample at 80°C for 12 min and plating on NYSM agar.

*Ultraviolet irradiation.* A distilled water suspension of sporulated cells of *B. laterosporus* NRS 590 ($1.7 \times 10^8$ spores/ml) was incubated in a 100-mm-diameter Petri dish on ice with constant stirring under an ultraviolet lamp ($4.0 \times 10^5$ ergs $cm^{-1}$ $sec^{-1}$). After 15 min of exposure, dilutions were made for plate counts and bioassay. Rifampicin and vancomycin were added to the bioassay cups at final concentrations of 0.005 and 1.0 μg/ml, respectively. These levels of antibiotics had been shown (unpubl.) to exceed the minimum inhibitory concentrations for *B. laterosporus* NRS 590 and did not themselves demonstrate any toxicity for the larvae.

*Preparation of culture supernatant.* Supernatant fluid from a 48-hr NYSM broth culture of NRS 590 was centrifuged at 70,000*g* for 2 hr and filtered through a 0.45-μm membrane filter, and the *p*H was adjusted to 7 before bioassay against *C. quinquefasciatus* larvae. Antibiotics were added to the bioassay to inhibit bacterial growth.

TABLE 1
PATHOGENICITY OF *Bacillus laterosporus* FOR *Culex quinquefasciatus* LARVAE[a]

| Strain | Spores/ml | $LC_{50}$ (μg/ml dry wt) | Source[b] |
|---|---|---|---|
| NRS 590 | $2.6 \times 10^8$ | 8.4 | ATCC |
| NRS 1111 | $3.7 \times 10^8$ | 14.4 | R. E. Gordon |
| NRS 1267 | $1.3 \times 10^8$ | 32.8 | R. E. Gordon |
| NRS 1645 | $3.3 \times 10^8$ | 14.0 | ATCC |
| NRS 1646 | $4.8 \times 10^8$ | 36.8 | ATCC |
| NRS 1647 | $5.5 \times 10^8$ | 29.3 | ATCC |
| NRS 1338 | $6.0 \times 10^8$ | 22.1 | R. E. Gordon |
| Bon 705 | $3.9 \times 10^5$ | 43.2 | G. J. Bonde |
| Bon 706 | $3.4 \times 10^2$ | 32.8 | G. J. Bonde |
| Bon 707 | $2.8 \times 10^7$ | 23.8 | G. J. Bonde |
| ATCC 64 | $2.0 \times 10^8$ | 10.5 | ATCC |
| ATCC 9141 | $3.3 \times 10^7$ | 13.0 | ATCC |
| ATCC 6457 | $4.9 \times 10^7$ | 10.3 | ATCC |
| Shi 1 | $2.7 \times 10^7$ | 28.5 | H. Shimanuki |
| Shi 2 | $6.9 \times 10^4$ | 12.7 | H. Shimanuki |
| Shi 5 | — | 63.8 | H. Shimanuki |
| Shi 3 | — | N.D.[c] | H. Shimanuki |
| Shi 4 | — | N.D. | H. Shimanuki |
| NRS 1642 | $2.1 \times 10^8$ | N.D. | ATCC |
| NRS 1643 | $2.2 \times 10^8$ | N.D. | ATCC |
| NRS 1644 | $1.6 \times 10^8$ | N.D. | ATCC |
| NRS 1648 | $3.2 \times 10^8$ | N.D. | ATCC |
| NRS 661 | $2.7 \times 10^6$ | N.D. | R. E. Gordon |
| NRS 882 | $1.6 \times 10^3$ | N.D. | R. E. Gordon |
| NRS 340 | $3.0 \times 10^2$ | N.D. | R. E. Gordon |
| Bon 708 | $3.2 \times 10^2$ | N.D. | G. J. Bonde |
| Bon 712 | $6.0 \times 10^2$ | N.D. | G. J. Bonde |
| CCEB 342 | $4.2 \times 10^8$ | N.D. | O. Lysenko |
| CCEB 629 | $3.0 \times 10^2$ | N.D. | O. Lysenko |

[a] All strains were grown in NYSM broth for 48 hr with shaking at 30°C.

[b] ATCC, American Type Culture Collection, Rockville, Md.; R. E. Gordon, Rutgers University, New Brunswick, N.J. (present address ATCC, Rockville, Md.); G. J. Bonde, Institute of Hygiene, University of Aarhus, Aarhus C, Denmark; H. Shimanuki, Bioenvironmental Bee Laboratory (USDA), Beltsville, Md.; O. Lysenko, Department of Insect Pathology, Institute of Entomology, CSAV, Flemingovo Nam 2, Prague 6, Czechoslovakia.

[c] N.D., not determinable. The percentage kill at the $10^{-1}$ dilution of the bacterial cell suspension was less than 50%.

*Heat treatment.* A distilled water suspension (10 ml) of sporulated cells of NRS 590

($1.7 \times 10^9$ spores/ml) was immersed in a water bath and heated at 96°C for 10 min.

*Preparation of a suspension of free spores.* A distilled water suspension of washed, 48-hr sporulated cells (9.67 mg dry wt/ml) of NRS 590 grown in NYSM broth was sonicated on ice for five 1-min intervals at a relative output of 0.55 using a Fisher sonic dismembrator Model 300 (Fisher Scientific, Pittsburgh, Penn.). To separate the spores from the cellular debris, the sonicated spore suspension was subjected sequentially to four 15-min cycles of centrifugation at 3840*g*, followed by one 15-min cycle of centrifugation at 1880*g* and, finally, two 15-min cycles of centrifugation at 640*g*. After each centrifugation, the supernatant which contained cellular debris was collected for bioassay and the spore-containing pellet was suspended in 150 ml of distilled water and recentrifuged. The spore-containing pellet of the final 640*g* centrifugation was suspended in 100 ml of distilled water. Three samples: the spore suspension which contained approximately 99% free spores lacking the sporangia, the pooled supernatants and the original sporulated cells were bioassayed.

*Cell fractionation.* A distilled water suspension of 8-hr stationary-phase cells (7.05 mg dry wt/ml) was sonicated on ice at a relative output of 0.95 (Fisher sonic dismembrator Model 300) for twenty 1-min intervals with cooling of the probe in ice between treatments. Approximately 90% of the cells were disrupted after the sonication. The sonicated cells were centrifuged at 48,400*g* for 20 min and the supernatant was collected. A portion of the supernatant was filtered through a 0.45-μm membrane filter and set aside for bioassay. The remaining unfiltered 48,400*g* supernatant fluid was centrifuged at 111,000*g* for 90 min. The supernatant resulting from this centrifugation was collected and filtered through a 0.45-μm membrane filter. The pellet from the 110,000*g* centrifugation was suspended in 20 ml of distilled water and filtered through a 0.45-μm membrane filter.

*Bioassays.* Toxic activity against mosquitoes was determined by assaying bacterial samples against second-instar larvae of *Culex quinquefasciatus* and *Aedes aegypti.* Ten second-instar larvae were suspended in a total volume of 20 ml of dechlorinated tap water in paper cups and 30 larvae were used per dilution. Ten cups, containing 10 larvae and 20 ml of dechlorinated tap water each, served as controls. Initially and at 48 hr a drop of 10% (w/v) debittered yeast was added to each cup. The larvae were held at 25°C for 72 hr when mortality was recorded. The $LC_{50}$ values were determined by the method of Hoskins (1967) with correction for control death using Abbots formula (Finney, 1952). The $LC_{50}$ is expressed in terms of the dry weight except in those instances when it is specifically stated to be based upon protein.

Pathogenicity of strains of *B. laterosporus* for larvae of the cabbage looper, *Trichoplusia ni,* was determined by placing 10 third-instar larvae into 100-mm-diameter plates containing Vanderzant–Adkisson wheat germ diet which had been smeared with 1 ml of a 1 mg/ml suspension of bacterial cells. A suspension (1 mg/ml) of *Bacillus thuringiensis* strain HD-1 served as a positive control. The percentage mortality was recorded each day for 3 days, at which time HD-1 had killed 100% of the larvae.

A lyophilized powder preparation of *B. laterosporus* NRS 590 (48-hr cells grown in NYSM broth) was assayed for pathogenic activity against tobacco budworm larvae (*Heliothis virescens*) by H. Dulmage (method of Dulmage et al., 1956) at the Cotton Insects Research Unit, U.S. Department of Agriculture, in Brownsville, Texas. This same lyophilized powder was also assayed for pathogenic activity against black fly larvae, *Simulium vittatum,* by D. Molloy (method of Gaugler et al., 1980) at the New York State Museum in Albany. Late-instar black fly larvae were exposed to the lyophilized powder at a concentration of 10 ppm/15 min.

*Microscopy.* To determine the average

size of exponential and stationary cells of NRS 590, a loopful of cells grown in NYSM broth was suspended in one drop of glycerol on a microscope slide. The length and width were determined using an ocular micrometer at 1000× magnification.

For electron microscopy of 4- and 8-hr cells of *B. laterosporus*, cell suspensions of strain NRS 590 were centrifuged and the pellet was suspended in ice-cold 4% glutaraldehyde in 0.17 M potassium phosphate buffer, *p*H 7.2. The cells were collected by centrifugation, suspended in tempered 2% agar, and cut into 1-mm cubes. The agar cubes were fixed in glutaraldehyde for 1 hr at 0°C, rinsed with 0.17 M phosphate buffered, *p*H 7.2, saline (0.7%), and placed for 1 hr in 1% osmium tetroxide made in the buffered saline. They were rinsed in distilled water, placed overnight in 0.5% uranyl acetate at 26°C, and dehydrated in ethanol before embedding in Epon 812. Sections were stained with 2% uranyl acetate and 0.1% alkaline lead citrate and examined by transmission electron microscopy.

*Protein and dry weight determinations.* Protein concentrations were determined by the method of Lowry et al. (1951). Protein was extracted from the bacteria by boiling in 1 N NaOH for 15 min. Dry weights were determined following heating of cells for 48 hr at 110°C.

## RESULTS

*Pathogenic activity of* B. laterosporus *for insects.* Twenty-nine strains of *B. laterosporus* were screened for pathogenicity to second-instar larvae of the mosquito, *C. quinquefasciatus* (Table 1). Sixteen of the strains demonstrated readily detectable pathogenicity. Most of the pathogenic strains produced a high number of heat-resistant spores ($>10^8$/ml) under the growth conditions used in this study. However, three of the pathogenic strains (Shi 2, Bon 705, and Bon 706) produced less than 4.0 $\times$ $10^5$ spores/ml.

In addition to screening for pathogenicity to mosquito larvae, all strains were tested for pathogenic activity to cabbage looper larvae. No pathogenicity was observed for any of the *B. laterosporus* strains, whereas the *B. thuringiensis* HD-1 suspension killed 100% of the larvae. No deaths occurred among the control larvae which were exposed to 1 ml of distilled water smeared onto their diet.

On the basis of repeated cultivation and bioassay of the mosquito larvicidal strains of *B. laterosporus*, one strain, NRS 590, was selected for further study. Larval diets containing either 500 μg/ml of an NRS 590 lyophilized powder preparation or a final whole culture caused mortalities of only 12 and 20%, respectively, when tested against *H. virescens* larvae. The lyophilized powder of NRS 590 was pathogenic to black fly larvae. However, it was approximately 100–1000 times less active than a primary powder of *B. thuringiensis* H-14.

In addition to assaying NRS 590 against larvae of the mosquito, *C. quinquefasciatus*, it was also assayed against larvae of *A. aegypti*. On the basis of a single determination there was very little difference in the toxic activity of NRS 590 for the two mosquitoes (14.3 and 14.4 μg/ml, respectively).

*Ultraviolet radiation treatment.* A sporulated cell suspension of NRS 590 was divided and one-half was exposed to UV irradiation. The UV treatment lowered the viable cell number by 7 logs. However, the nonirradiated sample and the UV-irradiated sample demonstrated similar $LC_{50}$ values (2.83 ± 1.03 and 2.51 ± 0.64 μg/ml, respectively). The possibility that pathogenicity was due to multiplication of surviving bacterial cells was eliminated by the addition of antibiotics to the assay cups.

*Toxic activity of the culture supernatant.* The culture supernatant fluid of NRS 590 failed to demonstrate toxic activity for mosquito larvae ($LC_{50}$ > 144 μg protein/ml). The cell mass was toxic ($LC_{50}$ = 1.13 ± 0.11 μg protein/ml).

*Heat stability of the toxin.* The number of viable spores of NRS 590 decreased from

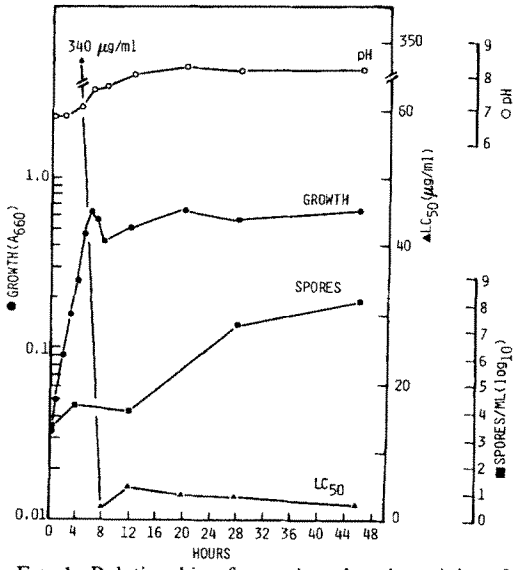


FIG. 1. Relationship of growth and toxic activity of *Bacillus laterosporus* NRS 590 grown in NYSM broth: (○) *p*H, (●) growth, (■) spores/ml, (▲) $LC_{50}$.

$1.7 \times 10^9$ to $2.7 \times 10^7$/ml after heating at 96°C for 10 min. However, the toxicity of the heated sample ($LC_{50}$ = 2.49 ± 0.71 μg/ml) changed very little compared to the nonheated sample ($LC_{50}$ = 2.03 ± 0.20 μg/ml).

*Growth cycle studies.* Since toxin synthesis by *B. thuringiensis* and *B. sphaericus* occurs during sporulation, experiments were conducted to determine whether *B. laterosporus* behaves similarly. During the exponential phase of growth (4 hr), the vegetative cell mass did not demonstrate any detectable toxicity for mosquito larvae (Fig. 1). However, by the early stationary phase (8 hr) the cell mass demonstrated full toxic activity. Since the $LC_{50}$ value is based on the dry weight of the cell mass, an increase in the number of cells producing an equivalent amount of toxin should not have changed the $LC_{50}$ value.

*Toxic activity of spores of strain NRS 590.* The failure of the cells to increase in toxicity as the spore and the associated parasporal body developed within the sporangium made it desirable to isolate these structures to determine if they were toxic. When a preparation containing 99% free spores with the attached parasporal bodies was bioassayed, it failed to demonstrate detectable toxic activity for mosquito larvae ($LC_{50}$ > 361 μg/ml). Both the original 48-hr sample from which the spores had been prepared and the pooled supernatants from the differential centrifugation were toxic. Thus, fully developed spores with associated parasporal bodies of NRS 590 do not represent major sites of toxic substance in this bacterium.

*Microscopic analysis of 4- and 8-hr cells of strain NRS 590.* When 4-hr (nontoxic) and 8-hr (toxic) cells were examined by phase-contrast microscopy, they appeared as normal vegetative cells with no obvious signs of sporulation. The typical swollen, fusiform sporangia did not appear until 20 to 24 hr after inoculation. The early stationary-phase cells, however, did appear to be shorter in length than the majority of the exponential-phase vegetative cells. The mean length of the 8-hr cells was 3.53 ± 0.87 μm ($n$ = 25) and the mean length of the 4-hr cells was 5.42 ± 1.47 μm ($n$ = 25). The mean width of the 8-hr cells was 0.96 ± 0.13 μm ($n$ = 25) and the mean width of the 4-hr cells was 1.17 ± 0.12 μm ($n$ = 25).

In addition to the size measurements, the 4- and 8-hr cells were examined by electron microscopy. Since the toxic activity of NRS 590 first appeared at 8 hr, it was desirable to determine if there were any distinct changes in the ultrastructure of the cells at that time. The 4-hr cells (Fig. 2) displayed divided nuclear material and a multilayered cell wall. None of the 8-hr cells (Fig. 3) contained structures normally associated with bacterial sporulation, e.g., a forespore septum. A unique feature seen in the 8-hr cells but not seen in any of the 4-hr cells was the presence of oval gray areas (GA) which were less granular in appearance than the cytoplasm (Fig. 3).

*Cell fractionation of NRS 590.* The toxicity of the various fractions obtained from NRS 590 is presented in Table 2. A sonicated 8-hr cell suspension (fraction 2) which contained cell fragments, a few

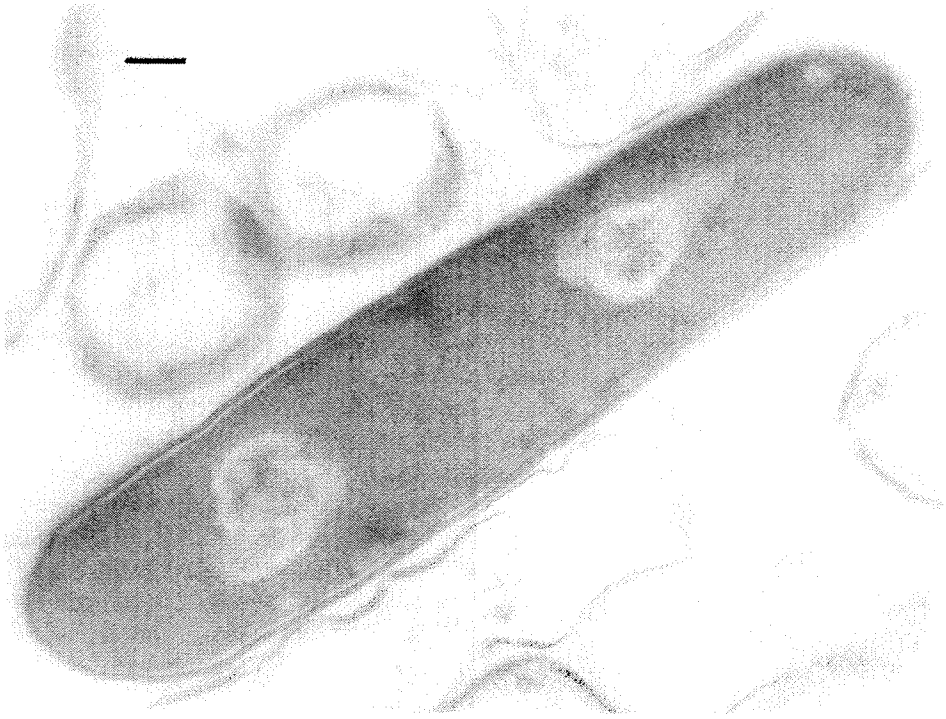

FIG. 2. Electron micrograph of a 4-hr (nontoxic) cell of *Bacillus laterosporus* NRS 590. Bar = 0.2 μm.

whole cells, and soluble cell material had a higher $LC_{50}$ than the original 8 hr cell suspension (fraction 1). When the sonicated cell suspension was fractionated into a cell particulate fraction (fraction 3) and a crude soluble fraction (fraction 4), the majority of the toxic activity was found with the particulate material rather than with the crude soluble fraction. The cell particulate fraction demonstrated a level of toxic activity that was equal to that demonstrated by the original 8-hr cells, whereas the crude soluble fraction was approximately 28 times less toxic than the original 8-hr cells. The ultracentrifugation of this crude soluble fraction produced a supernatant fraction (fraction 5) which failed to demonstrate any detectable toxic activity for mosquito larvae. The pellet (fraction 6) from the ultracentrifugation of the crude soluble fraction was toxic.

## DISCUSSION

Sixteen of the 29 strains of *B. laterosporus* were pathogenic to *C. quinquefasciatus* larvae, but none were pathogenic to *T. ni* larvae. This spectrum of activity is similar to that demonstrated by two other bacterial mosquito pathogens, *B. thuringiensis* serovar *israelensis* (H14) and *B. sphaericus*. However, the level of toxicity demonstrated for mosquito larvae by the most toxic strain of *B. laterosporus*, NRS 590, was about 1000 times less than the IPS-78 standard *B. thuringiensis* H14 powder as reported by Margalit et al. (1983). *B. laterosporus* NRS 590 was not pathogenic to larvae of the tobacco budworm, *H. virescens*, but it did demonstrate pathogenicity at high dosage toward larvae of the black fly, *S. vittatum*. It was equally toxic to larvae of the mosquitoes, *A. aegypti* and *C. quinquefasciatus*. In these respects, the insecticidal spectrum of strain NRS 590 is more like that of *B. thuringiensis* serovar *israelensis* (H14) than that of *B. sphaericus*. *B. sphaericus* is less toxic to *Aedes* larvae than to *Culex* larvae

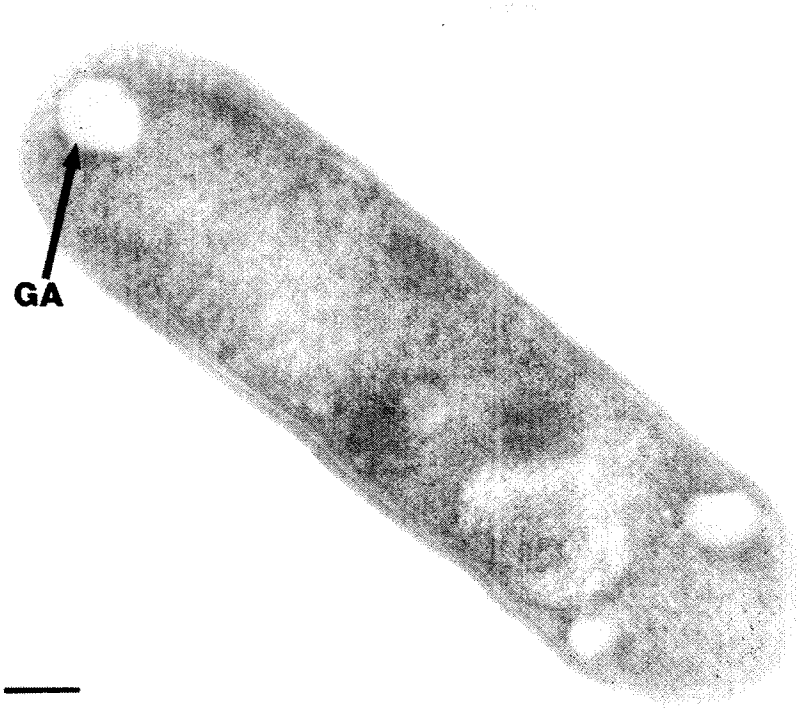


FIG. 3. Electron micrograph of an 8-hr (toxic) cell of *Bacillus laterosporus* NRS 590. Note the presence of the unique gray areas (GA) which were not seen in 4-hr cells. Bar = 0.2 μm.

and is not toxic to black fly larvae (Singer, 1980).

The pathogenic activity of *B. laterosporus* NRS 590 for mosquito larvae is not associated with the culture supernatant fluid but rather with the cell mass. This pathogenic activity is a toxin-mediated process because cells of NRS 590 killed by UV irradiation demonstrated larvicidal activity similar to that of nonirradiated cells. Also, a heated cell suspension in which the number of viable spores had decreased almost 100-fold demonstrated no loss in pathogenicity. In both of these treated samples, dead cells contributed to toxicity.

Growth cycle studies indicated that the toxic activity in NRS 590 was not associated with actively growing, vegetative cells and only appeared after the cells had ceased growing exponentially. The toxic activity was not associated with the heat-resistant spore itself or with the closely associated paraspore. When a preparation consisting of 99% spores and 1% sporangia was bioassayed, it was nontoxic to mosquito larvae. Growth cycle studies indicated that as the spores were formed, the toxicity of the cell mass remained constant. Thus, the formation of the spore structures, i.e., cortex, spore coats, and canoe-shaped parasporal body, did not increase the toxic activity of the cell mass. This further indicated that these spore structures were not sites of toxic activity.

Stationary-phase (8 hr) cells of *B. laterosporus* NRS 590 which were toxic to mosquito larvae displayed few differences in morphology when compared to nontoxic exponential-phase (4 hr) cells. The older cells were about 35% shorter than the younger, nontoxic cells. This is related to the transition from active cell growth to the

TABLE 2
TOXIC ACTIVITY OF CELL FRACTIONS OF NRS 590 FOR MOSQUITO LARVAE

| Fraction | | $LC_{50}$[e] | |
|---|---|---|---|
| | | Dry wt (μg/ml) | Protein (μg/ml) |
| 1 | Washed 8-hr cells[a] | 4.9 | 3.0 |
| 2 | Sonicated cell suspension | 60.4 | 30.5 |
| 3 | Sonicated cells and cell fragments[b] | 8.5 | 1.7 |
| 4 | 48,400*g*, filtered supernatant of the sonicated cells | | 85.7 |
| 5 | 111,000*g*, filtered supernatant (soluble)[c] | | >200.0 |
| 6 | 111,000*g* centrifugation pellet[d] | | 19.0 |

[a] Cells were grown in NYSM broth for 8 hr.

[b] This sample which represents the particulate fraction of the sonicated cell suspension was obtained by centrifuging the sonicated cells at 48,400*g* and collecting the pellet.

[c] This soluble fraction was obtained by centrifuging the 48,400*g* supernatant of the sonicated cells at 111,000*g*.

[d] This was the material which sedimented during the 111,000*g* centrifugation. This material was suspended in 20 ml of distilled water before bioassaying.

[e] The antibiotics rifampicin and vancomycin were added to the bioassay at a final concentration of 0.005 and 1.0 μg/ml, respectively. The data represent the results from an assay of one experiment.

stationary phase. It was particularly interesting that the toxic 8-hr cells lacked any morphological indications of spore development such as the forespore septum. This supports the conclusion that toxic activity is not associated with a particular spore structure. Thin sections of 8-hr cells revealed the presence of unidentified gray areas (GA) which were not present in exponential-phase cells. Whether these were related to toxicity was not determined.

Toxic activity in fractionated 8-hr cells of NRS 590 was associated with the particulate matter rather than with the soluble cytoplasmic material. The most likely sites for the toxin are the cell wall or the cytoplasmic membrane. In *B. sphaericus*, toxin was found associated with a cell wall fraction of sporulating cells (Myers and Yousten, 1980). However, in that bacterium toxicity has also been found in a fraction enriched in parasporal inclusions (Payne and Davidson, 1984). In contrast to the parasporal inclusions of *B. thuringiensis* and *B. sphaericus*, the canoe-shaped parasporal bodies of *B. laterosporus* do not seem to serve as sites of toxin localization.

The low levels of toxicity demonstrated by the strains of *B. laterosporus* in this study indicate that these strains are not potential candidates for biocontrol agents. However, additional strains of *B. laterosporus* may prove to be more toxic than the strains investigated in this study.

## ACKNOWLEDGMENTS

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# EXHIBIT G

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1989, p. 252–253
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Vol. 55, No. 1

# NOTES

## Expression in *Bacillus subtilis* of the 51- and 42-Kilodalton Mosquitocidal Toxin Genes of *Bacillus sphaericus*

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**A 3,080-base-pair *Kpn*I-*Hin*dIII DNA fragment from *Bacillus sphaericus* 2362 coding for 51- and 42-kilodalton mosquitocidal proteins was cloned into *Bacillus subtilis* DB104 by using the vector pUB18. In *B. subtilis* these proteins were not detected during vegetative growth but were expressed during sporulation at levels comparable to those found in *B. sphaericus*.**

In the course of sporulation *Bacillus sphaericus* 2362 produces a parasporal crystal which contains several proteins that are toxic to mosquito larvae (1, 3, 4). Recently, we cloned into *Escherichia coli* and sequenced a 3.5-kilobase (kb) DNA fragment which codes for toxin proteins of 51 and 42 kilodaltons (kDa) (1, 2). In *B. sphaericus* these genes are expressed only during sporulation at a final level corresponding to about 5% of the dry weight of the cells (4, 7), while in *E. coli* they are expressed at much lower levels throughout the growth cycle (2). Since both *Bacillus subtilis* and *B. sphaericus* form endospores, it is plausible that the *B. sphaericus* sporulation-associated expression of these proteins would also occur in *B. subtilis*. Recently, de Marsac et al. (5) have presented evidence for the expression of toxin genes from *B. sphaericus* 1593M in *B. subtilis*.

*B. subtilis* DB104 is a strain that is deficient in extracellular alkaline and neutral proteases (6). Plasmid pUB18 is a pUB110 derivative containing a M13mp18 multiple cloning site (8; T. H. Zaghloul, Ph.D. thesis, University of California, Davis, 1986). A 3.1-kb *Kpn*I-*Hin*dIII fragment (Fig. 1) from pGA-5 (2) was ligated into pUB18, and the preparation was used to transform *B. subtilis* DB104. The procedures used for the transformation of cells and plasmid miniscreening have been described previously (8). The resulting plasmid was designated pC7a. *B. subtilis* DB104 containing pC7a was grown at 37°C in medium containing a mineral base (4); 0.1% (wt/vol) D-glucose; and the following, per liter: 10 g of tryptone, 5 g of yeast extract, and 5 mg of kanamycin. Periodically, samples were removed, centrifuged, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were electroblotted onto nitrocellulose paper and detected by immunoblot assays by using antisera to all the crystal proteins or antisera which discriminated between the 42- and 51-kDa proteins. These procedures have been described previously in detail (3, 4).

A growth curve of *B. subtilis* DB104 containing pC7a is presented in Fig. 2. Exponential growth ceased about 2 h and 45 min after inoculation. After 8 h no significant decrease in turbidity was noted (tested up to 48 h). Figure 3 presents the kinetics of synthesis of the 51- and 42-kDa proteins. Neither protein was detected during exponential growth (lane a). The 42-kDa protein was first detected about 5 h after the cessation of exponential growth (lane d), while the 51-kDa protein was detected at 7 h after the cessation of exponential growth (lane e). Since the final level of the 42-kDa protein was greater than that of the 51-kDa protein, the difference in the time of their detection was probably a function of the difference in their amounts. The maximal level of the proteins was reached at or before 14 h after the inoculation of the culture (lane g) and remained approximately constant (lane h) up to 48 h (last time point sampled; data not shown) (lane i represents a sample taken at 36 h and contains twice the amount of cells present in lanes a to h). Degradation products of about 27 and 24 kDa were present in most of the samples containing the 42- and 51-kDa proteins. The concentration necessary to kill 50% of the second and third instar larvae of *Culex pipiens* ($LC_{50}$) was determined as described previously (3) by using the culture harvested at 36 h after the initiation of growth (lane i). The $LC_{50}$ was found to be 27 ng (dry weight) of cells per ml, as compared with 18 ng (dry weight) per ml found with a culture of *B. sphaericus* 2362 (3).

In *B. sphaericus* the amount of the 51- and 42-kDa proteins is approximately equivalently consistent with the suggestion that these two genes are in a single transcriptional operon (1). In *B. subtilis* the amount of the 51-kDa protein appeared to be considerably less than that of the 42-kDa protein. In order to test whether the 51-kDa protein is degraded to the 24- and 27-kDa peptides detected in Fig. 3, immunoblots of sample (lane g) were assayed by using antisera (3) which discriminated between the 42- and 51-kDa proteins. No major degradation products of the 51-kDa protein were detected (Fig. 4, lane b). The 24- and 27-kDa peptides had antigenic determinants of the 42-kDa protein (Fig. 4, lane c) and, therefore, appeared to be degradation products of this

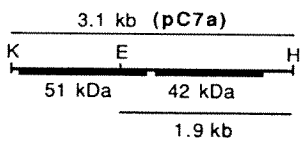


FIG. 1. Restriction map of the DNA fragments from *B. sphaericus* 2362 which were cloned into *B. subtilis* DB104. Abbreviations: E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I. Thick lines delineate the DNA coding for the toxin proteins.

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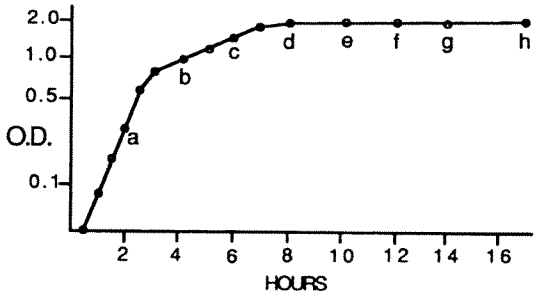


FIG. 2. Growth curve of *B. subtilis* DB104 containing pC7a. O.D., Optical density at 620 nm. Letters correspond to the times at which samples were removed (Fig. 3).

protein. We also tested the possibility that the 176-base-pair DNA fragment between the 51- and the 42-kDa proteins (Fig. 1) contained a promoter which functioned in *B. subtilis* and accounted for the greater amount of the 42-kDa protein. pC7a (Fig. 1) was cut with *Eco*RI (there was an *Eco*RI site in the mp18 multiple cloning site adjacent to the *Kpn*I site) and religated. The resulting plasmid contained a 1.9-kb *Eco*RI-*Hin*dIII DNA fragment, with the intact gene coding for the 42-kDa protein and the upstream region which included part of the gene coding for the 51-kDa protein (Fig. 1). In an experiment for which the results were analogous to those presented in Fig. 2 and 3, no cross-reacting material was detected in the samples (1 mg [dry weight] of cells) taken during exponential growth or at 24 and 36 h after inoculation of the culture (data not shown).

The results of this study indicate that in *B. subtilis* (Fig. 2 and 3), as in *B. sphaericus* (4), the 51- and 42-kDa proteins are expressed only in the course of sporulation and that the 96-base-pair DNA fragment between the *Kpn*I site and the initiation codon for the 51-kDa protein (Fig. 1) (1) contain all or most of the promoter region. This sporulation promoter appears to function in *B. subtilis* with an efficiency comparable to that of *B. sphaericus*, since the $LC_{50}$s of the two cell preparations for larvae of *C. pipiens* (an approximation of the amount of toxin) were similar. Unlike the case of *B.*

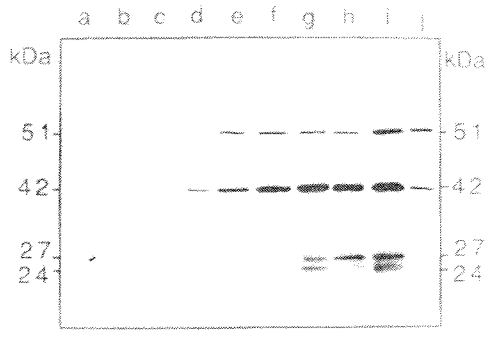


FIG. 3. Synthesis of the 51- and 42-kDa proteins during the growth cycle of *B. subtilis* DB104 containing pC7a. Lanes: a to h, 0.5 mg (dry weight) of cells, corresponding to the time points in Fig. 2 at which the samples were removed; i, 1.0 mg, sampled at 36 h after inoculation; j, 3 μg of crystal from *B. sphaericus* 2362. Lanes a to j, 8.5% acrylamide gel; proteins were detected on the immunoblot by using antisera to all the crystal proteins.

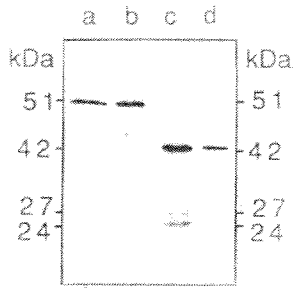


FIG. 4. Origin of the 24- and 27-kDa degradation products. Lanes a and d, 3 μg of crystal from *B. sphaericus* 2362; lane b, 1 mg of sample g (Fig. 3); lane c, 0.5 mg of sample g (Fig. 3). Proteins were detected on the immunoblot by antisera to the 42-kDa protein (lanes a and b) and antisera to the 51-kDa protein (lanes c and d); 10% acrylamide gels were used.

*sphaericus*, in which the amount of the 42- and 51-kDa proteins was about the same (4), the amount of detectable 42-kDa protein in *B. subtilis* was greater than that of the 51-kDa protein (Fig. 3). We have no explanation for this difference, which cannot, however, be readily accounted for by the degradation of the 51-kDa protein (Fig. 4) or the promoter activity of the DNA fragment between the genes coding for the two proteins.

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# EXHIBIT H

JOURNAL OF BACTERIOLOGY, Aug. 1989, p. 4178–4188
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# Cloning and Sequencing of the Gene Encoding a 125-Kilodalton Surface-Layer Protein from *Bacillus sphaericus* 2362 and of a Related Cryptic Gene

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**Using the vector pGEM-4-blue, a 4,251-base-pair DNA fragment containing the gene for the surface (S)-layer protein of *Bacillus sphaericus* 2362 was cloned into *Escherichia coli*. Determination of the nucleotide sequence indicated an open reading frame (ORF) coding for a protein of 1,176 amino acids with a molecular size of 125 kilodaltons (kDa). A protein of this size which reacted with antibody to the 122-kDa S-layer protein of *B. sphaericus* was detected in cells of *E. coli* containing the recombinant plasmid. Analysis of the deduced amino acid sequence indicated a highly hydrophobic N-terminal region which had the characteristics of a leader peptide. The first amino acid of the N-terminal sequence of the 122-kDa S-layer protein followed the predicted cleavage site of the leader peptide in the 125-kDa protein. A sequence characteristic of promoters expressed during vegetative growth was found within a 177-base-pair region upstream from the ORF coding for the 125-kDa protein. This putative promoter may account for the expression of this gene during the vegetative growth of *B. sphaericus* and *E. coli*. The gene for the 125-kDa protein was followed by an inverted repeat characteristic of teminators. Downstream from this gene (11.2 kilobases) was an ORF coding for a putative 80-kDa protein having a high sequence similarity to the 125-kDa protein. Evidence was presented indicating that this gene is cryptic.**

*Bacillus sphaericus* is a promising agent for the biological control of mosquitos which are vectors of important human and animal diseases (47). Toxicity for mosquito larvae has been associated with the formation, during sporulation, of a parasporal crystal and with proteins of 100 to 125 kilodaltons (kDa) which may be made during vegetative growth (3, 4, 7, 9, 12, 32, 33). In the case of *B. sphaericus* 2362, proteins with a molecular size of 42, 51, and 110 kDa have been shown to play a role in toxicity for mosquito larvae (2–4, 9). The genes for the 42- and 51-kDa proteins have recently been cloned and sequenced (2, 3, 21).

A number of gram-positive and gram-negative bacteria possess a protein or glycoprotein surface (S) layer that forms a barrier between the cell and the environment (39, 40). These proteins may constitute between 5 and 10% of the total protein of cells in exponential growth (39, 40). The high energy expenditure required for the synthesis of such a large amount of protein suggests that it has a vital function requiring its maintenance on the cell surface. Evidence has been presented indicating that the S layer acts as a protective barrier and plays a role in bacterial pathogenesis (22, 40). In the larvicidal strains of *B. sphaericus*, the S layer consists of a linear array of glycoproteins (29), the monomer having a molecular size of 127 to 129 kDa (45). In this species, it may serve as a site for bacteriophage attachment (28). On the basis of susceptibility to different bacteriophages, the mosquito-pathogenic strains have been subdivided into groups (47) which are in agreement with the subdivisions established by serological studies of the S-layer protein (29, 45).

In the present study, we cloned and sequenced a gene which codes for a 125-kDa precursor of the 122-kDa *B. sphaericus* 2362 S-layer protein (gene 125). Evidence is also presented indicating that the 122-kDa protein is the precursor of the 110-kDa larvicide and that both of these proteins are absent from the parasporal crystal of this species. The latter finding indicates that our previous conclusion that the 122- and 110-kDa proteins were constituents of the crystal is invalid since our "crystal" preparation (4) appears to have been contaminated with cell wall material containing S-layer proteins. The cryptic gene also cloned and sequenced in this study which codes for a putative 80-kDa protein has been designated as gene 80.

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## MATERIALS AND METHODS

**Bacterial strains and vectors.** *B. sphaericus* 2362 was used in our previous investigations (2, 4, 9). The λ vector EMBL3 (15) with the host strains *Escherichia coli* NM538 and NM539, the λ vector GEM2 (34) with the host strain *E. coli* LE392, and pGEM-4-blue were obtained from Promega Biotec (Madison, Wis.). The sources of *E. coli* TB1, the host for pGEM-4-blue, as well as *E. coli* JM101 and JM107 (31), used as the host strains for M13mp18/19, have been previously indicated (2, 3).

**General procedures.** Methods for restriction endonuclease analysis of DNA, agarose gel electrophoresis, large-scale λ DNA purification, plasmid DNA purification by $CsCl_2$-ethidium bromide density gradient centrifugation, and minipreparations of plasmid and bacteriophage DNA have been described by Maniatis et al. (30). Transformation of competent cells by plasmid DNA was performed by the procedure of Hanahan (18). Enzymes and substrates purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), New England BioLabs, Inc. (Beverly, Mass.), and Promega Biotec were used according to the directions of the manufacturers. The following procedures were performed as previously described (2, 4): sodium dodecyl sulfate-poly-

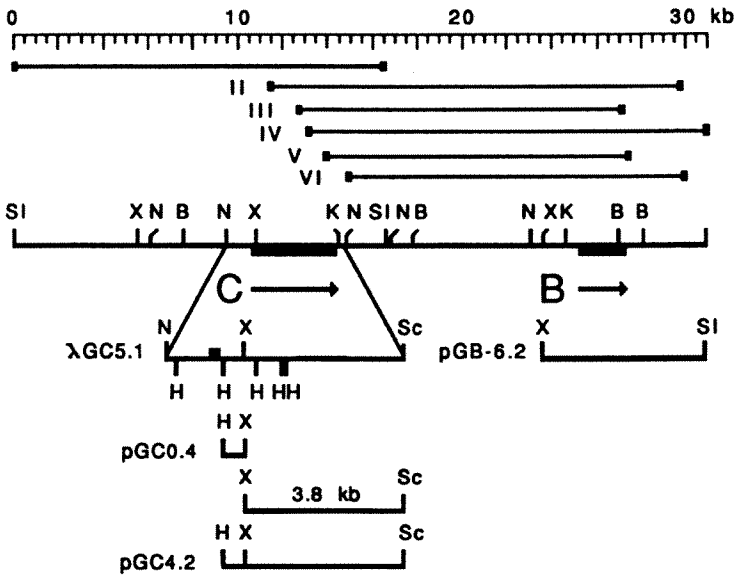



FIG. 1. Composite restriction map of a 32-kb fragment of *B. sphaericus* DNA containing gene 125 and gene 80 and the strategy used for subcloning these genes. Stippled bars indicate the regions coding for proteins. Arrows indicate direction of ORF. Regions spanned by bars and designated by Roman numerals represent the classes of λEMBL3 recombinants. Black bar on λGC5.1 indicates the region of the DNA which could not be subcloned into *E. coli*. B, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; N, *Nhe*I; Sc, *Sac*I; Sl, *Sal*I; X, *Xba*I.

acrylamide gel electrophoresis (SDS-PAGE), electroblotting of proteins onto nitrocellulose and detection with antiserum to the crystal proteins (Western immunoblots), preparation of cell extracts of *E. coli*, Ouchterlony immunodiffusion experiments (antiserum dilution of 1:4), and bioassays involving larvae of the mosquito *Culex pipiens*. Protein content was determined by the BCA Protein Assay (Pierce Chemical Co., Rockford, Ill.), with bovine serum albumin as the standard.

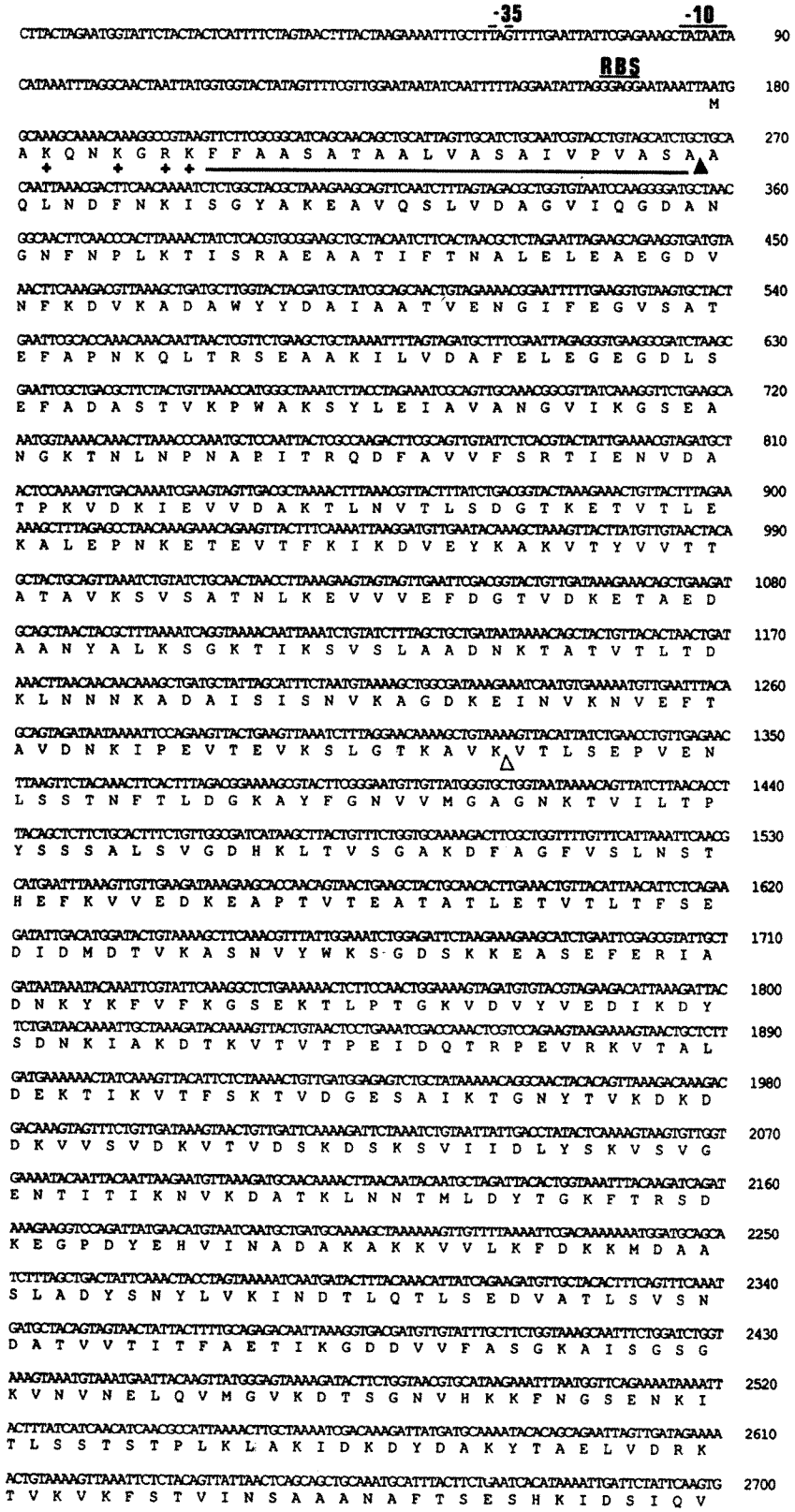
**Cloning of gene 80.** *B. sphaericus* 2362 DNA was isolated (2), partially digested with the restriction enzyme *Sau*3A, and size fractionated on sucrose gradients (30). The 10- to 20-kilobase (kb) *Sau*3A-digested DNA fragments were ligated into the *Bam*HI-digested λEMBL3 vector (15, 23), packaged by means of the Packagene Lambda DNA System (Promega Biotec), and plated on *E. coli* NM538 ($Spi^+$, permissive strain) and NM539 ($Spi^-$, nonpermissive strain) (23). A 0.5-ml portion of the packaging mix contained a total of $2.4 \times 10^5$ PFU; 41% of the phage particles were recombinants.

The library was screened by plating approximately 1,000 recombinant PFU per plate on *E. coli* NM539 (23). Plaque lifts were made with nitrocellulose filter disks (6). $^{35}S$-labeled DNA probes of gene 80 and gene 125 (B and C genes, respectively, in reference 2) were obtained as described previously (2). A mixture of the radioactive gene 80 and gene 125 probes was hybridized to nitrocellulose filters under conditions of high stringency (5°C less than the temperature at which 50% of the DNA double helix is dissociated). At this temperature there was no cross hybridization between gene 80 and gene 125 (30). X-ray film (AR; Eastman Kodak Co., Rochester, N.Y.) was exposed to the filters as directed by the manufacturer. Recombinant phage which hybridized with the mixture of gene 80 and gene 125 probes were plaque purified three times. A total of $2.3 \times 10^4$ recombinant λEMBL3 phage yielded 24 positive plaques. These were characterized by restriction enzyme digestion followed by Southern blotting and hybridization with probes to either gene 80 or gene 125 as previously described (2) (Fig. 1). Six classes of overlapping recombinants were obtained (Fig. 1), with each class represented by the following number of identical recombinants; I = 6, II = 1, III = 6, IV = 4, V = 6, and VI = 1.

Using the *Sal*I site located in the polylinker of λEMBL3, we subcloned the 6.2-kb *Xba*I-*Sal*I fragment from the λEMBL3 class VI recombinant (Fig. 1), which contained the DNA hybridizing with the gene 80 probe, into pGEM-4-blue and designated it pGB6.2 (Fig. 1).

**Cloning of gene 125.** Difficulty was encountered in routinely obtaining sufficient DNA from the λEMBL3 class I recombinants for the subcloning of gene 125 since the titers of the recombinant phage were generally below $5 \times 10^8$ PFU/ml of broth culture as compared with $10^{10}$ to $10^{11}$ for the remaining λEMBL3 recombinants. Based on the restriction enzyme analysis (Fig. 1), a different strategy was devised for obtaining gene 125. The DNA from *B. sphaericus* 2362 was cut with *Nhe*I-*Sac*I (Fig. 1), ligated into λGEM2, packaged as described above, and plated on *E. coli* LE392. The titer of the resulting preparation was $3 \times 10^6$ PFU in 0.5 ml, and approximately 80% of the phage contained recombinants. The resulting library was screened for hybridization with the gene 125 probe as described above; 28 positive plaques were obtained from $3.6 \times 10^4$ recombinants. All were characterized by restriction analysis and found to be identical. This λGEM2 recombinant was designated λGC5.1 (Fig. 1).

Subcloning of a smaller DNA fragment containing gene 125 from λGC5.1 proved to be a problem since it was not possible to obtain any recombinants in pGEM-4-blue which contained DNA 150 base pairs upstream of the second *Hin*dIII site (Fig. 1). A similar result was obtained by Yamagata et al. (46), and Belland and Trust (5). Yamagata et al. (46) were unable to clone the 5′ region of a related cell wall-associated protein gene (see Discussion) from *Bacillus*



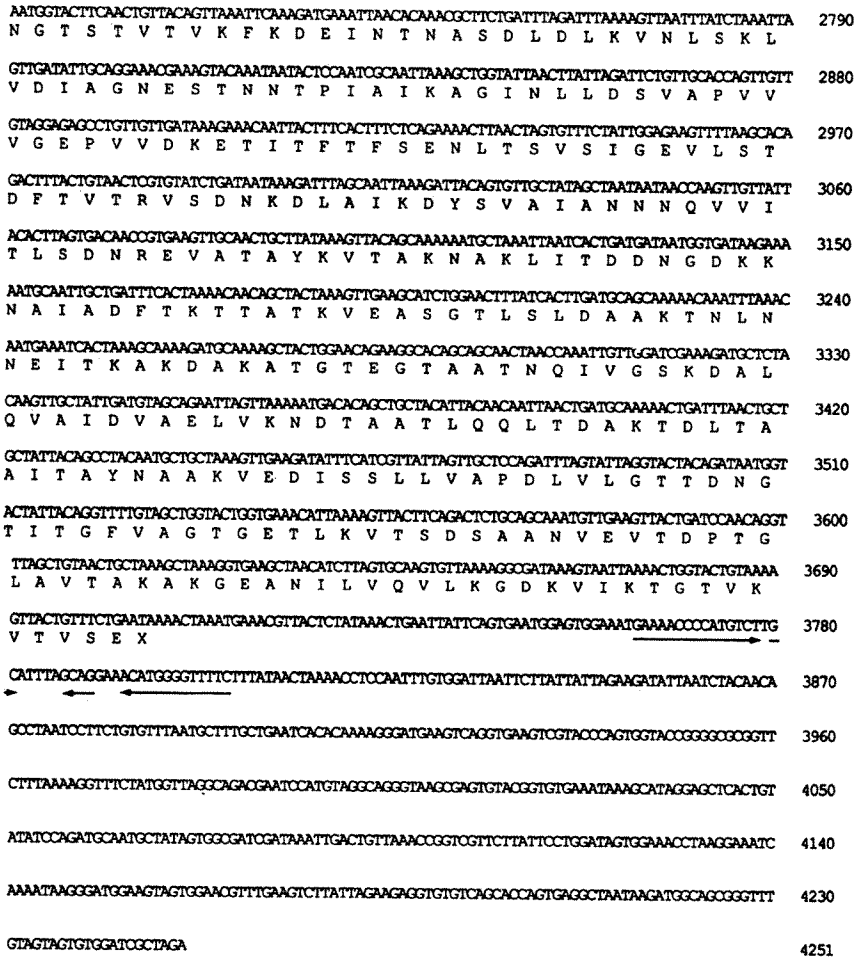



FIG. 2. Nucleotide sequence of the 4,251-base-pair pGC4.2 *B. sphaericus* DNA insert containing gene 125. The predicted amino acid sequence is given by the single-letter code. Lines labeled −35 and −10 designate the putative regions recognized by primary vegetative RNA polymerase. RBS, Putative ribosome-binding site; +, charged amino acid residues of the leader peptide; underlined amino acids, hydrophobic region of the leader peptide; ▲, the site of cleavage of the signal peptide; △, the beginning of the sequence of the related gene 80 ORF; arrows, inverted repeat.

*brevis* 47 into *E. coli*. Belland and Trust (5) were unable to subclone the 5′ region of the S-layer protein gene from *Aeromonas salmonicida* from a λgt11 clone into pBR322 or pUC18. Gene 125 was therefore reconstructed by first subcloning a 0.4-kb *Hin*dIII-*Xba*I fragment into pGEM-4-blue to give the recombinant pGC0.4 (Fig. 1). This plasmid was cut with *Xba*I and *Sac*I and ligated to the 3.8-kb *Xba*I-*Sac*I fragment from λGC5.1 (Fig. 1) to give the recombinant pGC4.2.

**DNA sequencing.** A more detailed restriction enzyme analysis was performed on the inserts of plasmids pGC4.2 (gene 125) and pGB6.2 (gene 80) (data not shown). Overlapping 1 to 2 kb DNA fragments were subcloned into M13mp18 and -19 by standard methods (31). A series of overlapping deletions were obtained with the IBI Cyclone System (International Biotechnologies, Inc., New Haven, Conn.) (11). In all cases, both strands of the DNA were sequenced by the dideoxy-chain termination method (37) with [$^{35}$S]dATP (Amersham Corp., Arlington Heights, Ill.) as the radioactive label.

**Purification of 122- and 110-kDa proteins.** The 122-kDa S-layer protein used for N-terminal amino acid sequence determination and immunological studies was purified as follows. Exponentially growing cells of *B. sphaericus* 2362 were harvested at an optical density at 620 nm of 0.2, and the cell walls were purified as described by Lewis et al. (29). The cell wall preparation was layered on a 48% NaBr solution

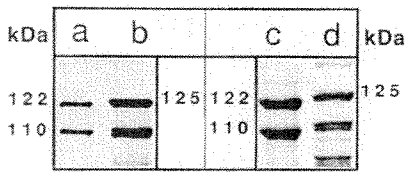



FIG. 3. Comparison of the gene 125 products made in *E. coli* by pGC4.2 with the 122- and 110-kDa S-layer proteins in the crystal preparation obtained from sporulated cells of *B. sphaericus* 2362. Lanes a and b, Western immunoblots, SDS-PAGE (7%); lanes c and d, SDS-PAGE (6%) stained with Coomassie blue. Lane a, 4 μg of crystal preparation; lane b, 30 μg of *E. coli* cell extract; lane c, 50 μg of crystal preparation; lane d, 100 μg of *E. coli* cell extract.

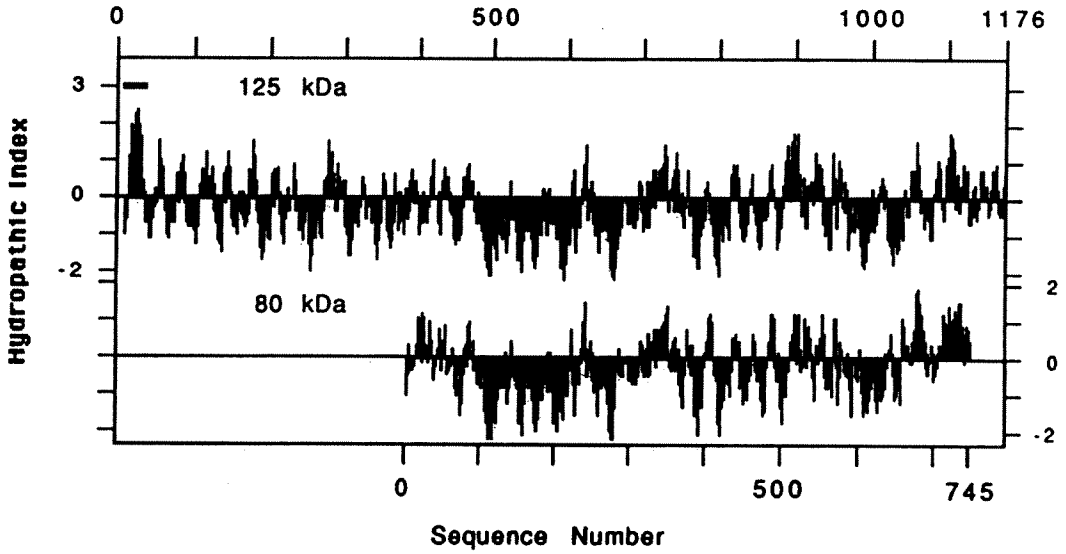



FIG. 4. Hydropathic analysis of the deduced amino acid sequence of the 125-kDa protein and the putative 80-kDa protein. The plots are aligned by the identical sequences. Solid bar indicates the hydrophobic region of the leader peptide.

and centrifuged at 17,000 × *g* for 3 h at 22°C in an HB-4 swinging-bucket rotor (Sorvall-DuPont, Newton, Conn.). The single top band contained the S-layer protein associated with the cell wall fragments. The purified cell wall suspension was dialyzed against water, and the S-layer protein was solubilized by treatment with 6 M urea (29) followed by dialysis against 10 mM $NaH_2$-$Na_2HPO_4$ buffer (pH 7.0). About 2 mg of the S-layer protein was obtained from a 1-liter culture.

The 122-kDa S-layer protein used for antiserum preparation was purified as described above with the sole difference that it was eluted from a nondenaturing polyacrylamide gel and dialyzed against 0.7% (wt/vol) NaCl.

The 122- and 110-kDa proteins from the crystal preparation (4) which were used for N-terminal amino acid sequence determination and immunological studies were purified by separation on SDS-PAGE (5% acrylamide) (4). The two regions of the gel containing the proteins were excised, and the proteins were electroeluted from the gel slice by use of the Elutrap Chamber (Schleicher & Schuell, Inc., Keene, N.H.) according to the directions of the manufacturer. SDS was removed by acetone precipitation (16), and the protein was dialyzed against 10 mM $NaH_2$-$Na_2HPO_4$ buffer (pH 7.0).

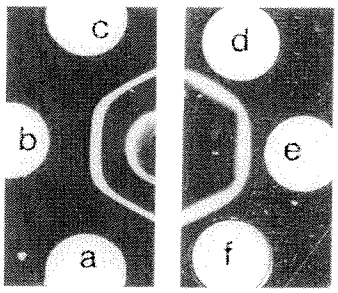



FIG. 5. Relationships of the 122- and 110-kDa proteins obtained from the crystal preparation, recombinant-produced 125-kDa protein, and the 122-kDa S-layer protein established by Ouchterlony immunodiffusion experiments. (a and d) cell-extract (200 μg) of *E. coli* containing pGC4.2 (125 kDa); (b and f) 20 μg of 122-kDa protein from crystal preparation; (c) 20 μg of 122-kDa S-layer protein; and (e) 20 μg of previously purified (9) 110-kDa protein. Center well, Anti-SC.

**N-terminal sequence determination.** With samples of 200 to 500 μg, the N-terminal sequences of the 122- and 110-kDa proteins from the crystal preparation and of the 122-kDa S-layer protein were determined by automated Edman degradation at the Protein Structure Laboratory of the University of California, Davis, School of Medicine (4).

**Antisera.** In this study, we used two antisera, of which the first was against a mixture of *B. sphaericus* 2362 S-layer and crystal proteins (122, 110, 51, and 42 kDa) (4, 9) (anti-SC), while the second was only against the 122-kDa S-layer protein (anti-S) from the same strain. For the latter, the antiserum was obtained from rabbits immunized as described by Lewis et al. (29). In the initial experiments involving 125-, 122-, and 110-kDa proteins, anti-SC was used. Subsequently selected experiments were confirmed by use of anti-S.

**Electron microscopy.** *B. sphaericus* 2362 was harvested during the exponential phase of growth at 30°C in NYSM broth (29) as well as from an overnight culture. The cells in 1.5 ml of the culture were suspended in 2% glutaraldehyde in 0.1 M $NaH_2$-$Na_2HPO_4$ (pH 7.0), stored at 4°C for 5 h, and washed twice in the buffer without glutaraldehyde. The preparation was suspended in 1% agar at 45°C and immediately centrifuged as the agar solidified. The agar containing the cells was cut into cubes and dehydrated in successive 10-min incubations in 30, 50, 80, 95, and 100% ethanol (the last performed three times). The samples were infiltrated overnight with a 1:1 Lowicryl K4M-ethanol mixture, for 8 h with a 3:1 Lowicryl-ethanol mixture, and for 4 days in 100% Lowicryl (all at 4°C). The infiltrated samples were polymerized under UV light for 3 days at room temperature, and sections were collected on nickel grids. Immunostaining was done by treatment with buffer A (20 mM Tris hydrochloride [pH 7.4], 500 mM NaCl, 0.3% Tween 80, 1% [wt/vol] bovine

serum albumin) for 10 min at room temperature followed by 2 h of incubation in anti-S (29) diluted 1/100 in buffer A. Grids were washed by immersion in buffer A lacking the albumin. They were subsequently incubated for 1.5 h in a solution containing *Staphylococcus aureus* protein A conjugated with 5 nM colloidal gold which had been diluted to a pale pink color in buffer A. The grids were washed twice in buffer A lacking albumin and twice in distilled water. Sections were poststained for 10 min in 1% (wt/vol) aqueous uranyl acetate and for 10 s in lead citrate.

## RESULTS

**Restriction analysis of DNA fragment containing gene 80 and gene 125.** The restriction enzyme analysis of the λEMBL3 recombinants is presented in Fig. 1. The positions of gene 125 and gene 80 are indicated together with the DNA fragments which were subcloned into the plasmid vector pGEM-4-blue. The two genes were found to be within 11.2 kb of each other and were localized in a 32-kb DNA fragment.

**Properties of gene 125.** The sequence of a 4,251-base-pair DNA fragment containing gene 125 was determined (Fig. 2). This fragment contained an open reading frame (ORF) of 3,528 nucleotides (nt), beginning at nt 178 and ending at nt 3,705, which coded for a protein of 1,176 amino acids with a deduced molecular size of 125,085 daltons. The coding region is preceded by the sequence GGAGG (at nt 163) characteristic of a ribosome-binding site. The 12-nt spacing between the middle A of this sequence and the ATG initiation codon, as well as the free energy of binding (41) between this region of *B. sphaericus* mRNA and its counterpart in the *B. subtilis* 16S rRNA (−14.4 kcal/mol) is typical of that found in gram-positive bacteria (17). Beginning at nt 84 (Fig. 2) there is a putative −10 sequence, TATAAT, corresponding to the consensus −10 sequence utilized by the primary vegetative sigma factor of *Bacillus subtilis* and *E. coli* (13). The putative −35 sequence at nt 59 has the highly conserved TntG of the consensus TTGACA sequence and maintains the optimal 18-base-pair spacing (19). Following the ORF at nt 3,763 to 3,806 is an inverted repeat which is complementary in 17 of 19 nt having −29.2 kcal/mol as the free energy of binding (41). This G+C-rich hairpin loop is suggestive of a transcription termination signal.

**Relation of 125-kDa protein to 122- and 110-kDa proteins.** Figure 3 compares the migration of the 122- and 110-kDa proteins from the crystal preparation in SDS-PAGE and Western immunoblots with the proteins synthesized in *E. coli* TB1 containing the recombinant plasmid pGC4.2. The largest recombinant-produced protein (125 kDa) (lanes b and d) had a larger molecular size than the 122-kDa protein from the crystal preparation (lanes a and c). In *E. coli*, some degradation products (lane b) primarily consisting of proteins of 112 to 114 kDa and 95 kDa were present. No detectable difference was observed in the amount of the 125-kDa protein or degradation products in *E. coli* cells harvested from the exponential or stationary phase of growth (data not shown).

The results of a hydropathic analysis by the computerized method of Kyte and Doolittle (26) are presented in Fig. 4. The N-terminus of the 125-kDa protein is highly hydrophobic. Examination of the primary sequence indicates that the first 30 residues (Fig. 2) have characteristics of a leader peptide found in secreted proteins of gram-positive organisms (10). Among these properties are four positively charged residues followed by a hydrophobic stretch of 21

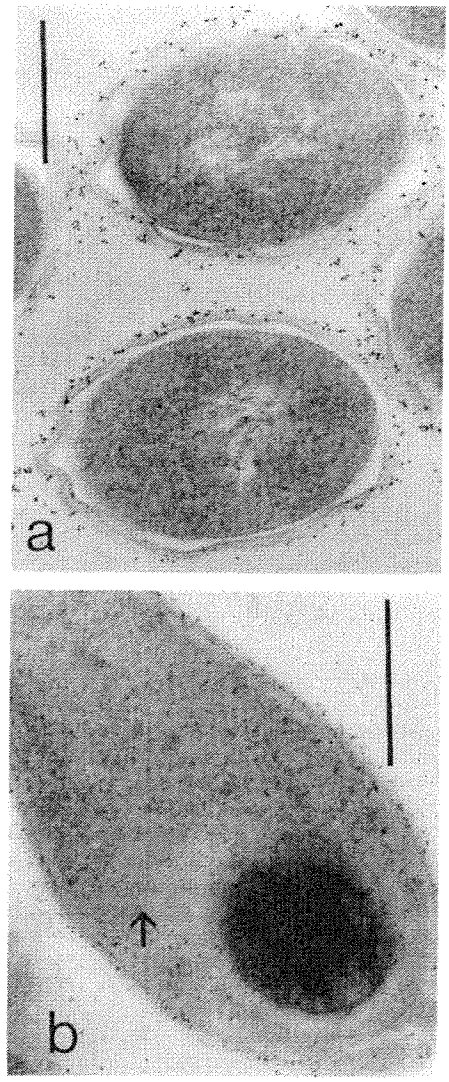



FIG. 6. Immunogold staining of thin sections of *B. sphaericus* 2362 with anti-S. (a) Cells in the exponential phase of growth. (b) Cells in the process of sporulation. Arrow in panel b designates the parasporal crystal. Bar, 0.5 μm.

amino acids which at its end contains the potential cleavage signal sequence ASA. The 12 N-terminal amino acids of SDS-PAGE-purified 122-kDa protein from the S layer and 122- and 110-kDa proteins from the crystal preparation were determined and found to be identical (AQLNDFNKISGY). This is the sequence of the 125-kDa protein following the signal sequence ASA (Fig. 2, nt 267). The molecular size of the 125-kDa protein with the leader peptide removed is 122,129 daltons.

The detection of a 125-kDa protein in *E. coli* cells containing the recombinant plasmid pGC4.2 (Fig. 3, lanes b and d) suggests that the leader peptide is not functional in this species. This is consistent with our inability to detect protein reacting with anti-SC in the supernatant fraction of the culture (tested by Western immunoblots, data not shown).

Ouchterlony immunodiffusion experiments (Fig. 5) showed a reaction of identity between the 125-kDa protein made in

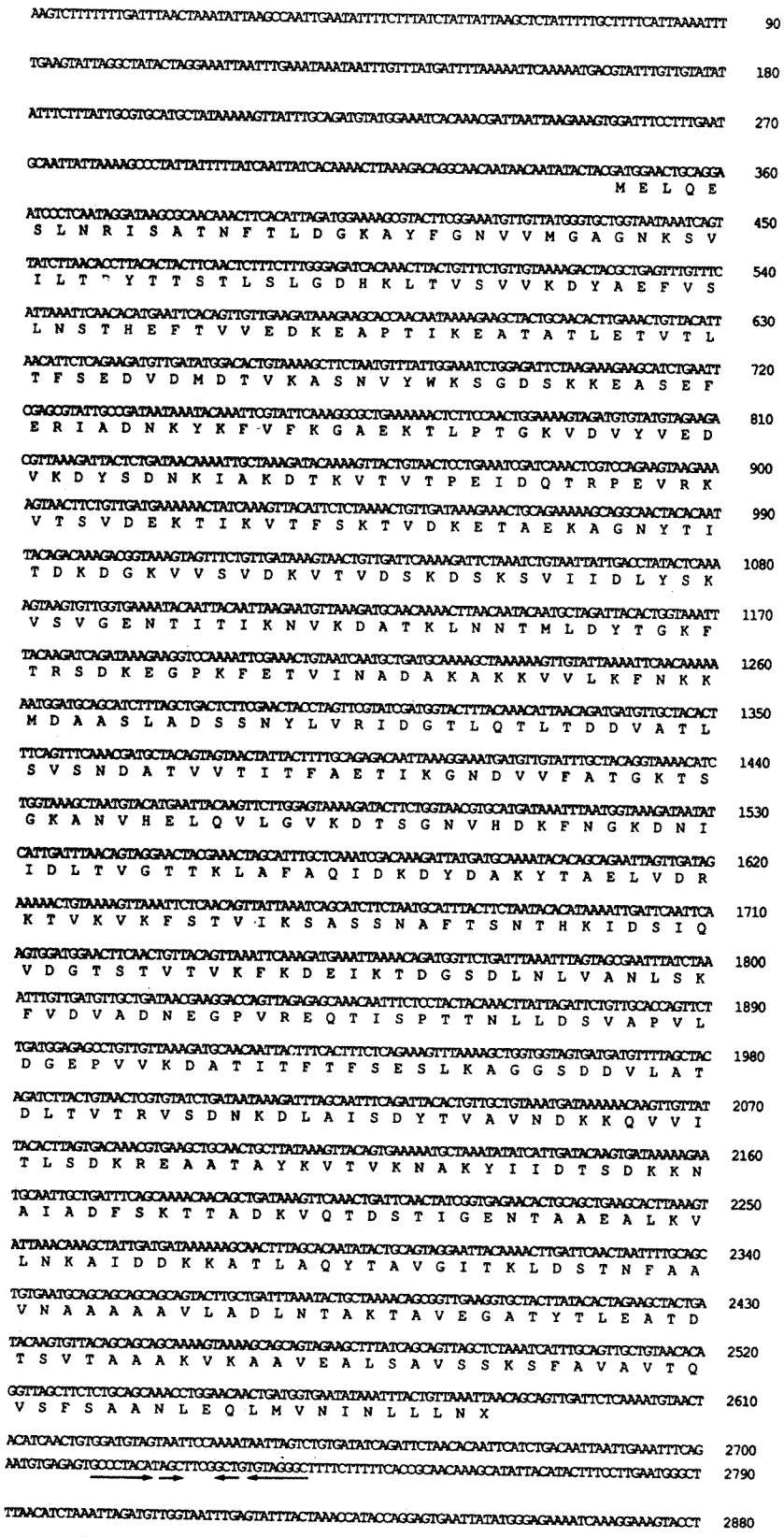


FIG. 7. Nucleotide sequence of the 2,880-base-pair region of *B. sphaericus* DNA containing gene 80. The predicted amino acid sequence is given in the single-letter code. Arrows designate an inverted repeat.

*E. coli*, the 122-kDa protein purified from the crystal preparation, the 122-kDa protein purified from the S layer, and the 110-kDa protein previously purified from the crystal preparation and shown to be toxic for larvae of *C. pipiens* (9). The results of these experiments are consistent with sequence identity or a high sequence similarity among these proteins. In addition, the identity of the N-terminal sequences of the 122- and 110-kDa proteins indicates that the former is the precursor of the latter and that the reduction in the molecular size is due to removal of amino acids at the C terminus.

**Electron microscopy.** Thin sections of *B. sphaericus* 2362 in the exponential phase of growth (Fig. 6a) or during the process of sporulation (Fig. 6b) reacted with anti-S predominantly at the region of the cell corresponding to the location of the S-layer protein. Anti-S did not react with the parasporal crystal (Fig. 6b), indicating that the 122- and 110-kDa proteins are not associated with the crystal.

**Toxicity of 125- and 122-kDa proteins.** Using second- to third-instar larvae of *C. pipiens*, we assayed the toxicity of *E. coli* cells containing pGC4.2 which accumulate the 125-kDa protein and proteins of 110 to 113 kDa (Fig. 3, lanes b and d). No toxicity was found up to a concentration of 67 μg (dry weight) of cells per ml. The purified cell wall fraction from exponentially growing cells of *B. sphaericus* 2362, which by SDS-PAGE was shown to contain only the 122-kDa S-layer protein, was also assayed. No toxicity was detected at a concentration of up to 20 μg of protein per ml.

**Properties of gene 80.** The sequence of a 2,880-base-pair DNA fragment containing gene 80 (Fig. 1) was determined (Fig. 7). One major ORF was detected (nt 346 to 2,580) which could code for a protein of 745 amino acids with a deduced molecular size of 80,024 daltons. The putative protein was not preceded by a recognizable ribosome-binding sequence. Following the ORF at nt 2,712 to 2,739 is an inverted repeat in which 11 of the 12 nt are complementary. The free energy of binding between these nucleotides was −22 kcal/mol (41). This hairpin structure together with the following row of Ts is suggestive of a transcription termination signal.

The amino acid sequence of the putative 80-kDa protein has extensive homology to that of the 122-kDa protein as indicated by the dot-matrix comparison presented in Fig. 8 (MacGene Plus; Applied Genetic Technology, Inc., Fairview Park, Ohio). Except for a portion of 127 amino acids at the C terminus, the similarity extends throughout the molecule. The region between amino acids 13 and 618 of the putative 80-kDa protein (Fig. 7) has an 81% sequence similarity to the region between amino acids 365 and 974 of the 122-kDa protein (Fig. 2). The sole changes required for this comparison were the introduction into the putative 80-kDa protein sequence of a 3-amino-acid gap at residue 364 and a 1-amino-acid gap at residue 505 (Fig. 7). Comparison of the nucleotide sequence within the same region indicated a similarity of 88%. A hydropathic analysis of the 80-kDa protein is presented in Fig. 4. The results showed a major similarity in the hydrophobicity profiles of the 80- and 125-kDa proteins, with differences residing at the C termini of these two molecules.

Western immunoblots indicate that the gene coding for the putative 80-kDa protein, contained on pGB6.2 (Fig. 1), is not expressed in *E. coli* (data not shown). Similarly, this fragment (Fig. 1) when cloned into *B. subtilis* DB104 or *B. sphaericus* SSII-1 did not result in the production of a detectable protein in Western immunoblots, in contrast to the DNA fragment contained on pGC4.2 (Fig. 1), which when cloned into these species produced the 122-kDa gene

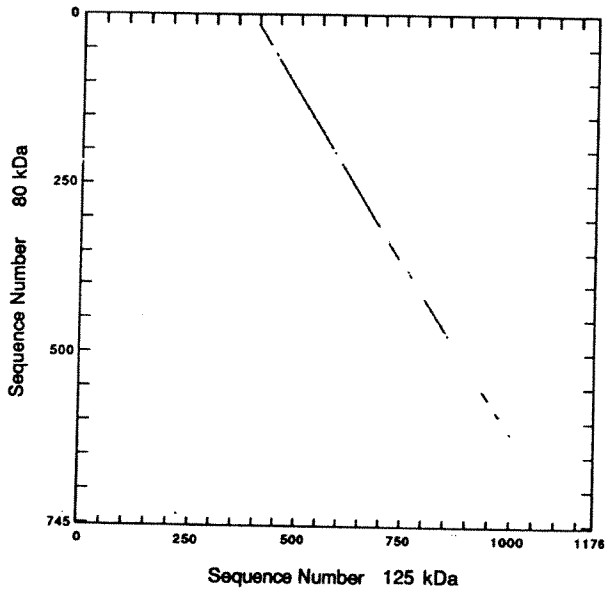



FIG. 8. Comparison of the 125-kDa protein and the putative 80-kDa protein by a dot-matrix plot. The window was 10 amino acids, and the stringency was 90%.

product (R. D. Bowditch, Ph.D. thesis, University of California, Davis, 1989). These results and the absence of a discrete 80-kDa protein band in Western immunoblots of vegetative and sporulating cells of *B. sphaericus* 2362 leads to the conclusion that the gene coding for the 80-kDa protein is cryptic.

**Codon usage.** Table 1 compiles the codon usage for the genes coding for the 42- and 51-kDa crystal proteins, as well as the 125-kDa S-layer protein precursor of *B. sphaericus* 2362. The codon usages for these three genes are compared with those of *E. coli* and *B. subtilis* (36). As would be expected from the low guanine-plus-cytosine content of the DNA of *B. sphaericus* (25) (35 mol%), there is generally a strong bias for A or U in the third position.

## DISCUSSION

The results of this study indicate that gene 125 codes for a 125-kDa protein which contains a leader peptide (Fig. 2) and is the precursor of the 122-kDa S-layer protein. The latter is the precursor of the 110-kDa larvicidal protein (9) which appears during sporulation of *B. sphaericus* 2362. This conclusion is based on the reaction of identity among these proteins in Ouchterlony immunodiffusion experiments (Fig. 5), the sequence identity of the N-terminal portion of these molecules, and the reaction of anti-S with the cell wall but not the parasporal crystal (Fig. 6) of *B. sphaericus* 2362.

In *B. sphaericus*, the S-layer protein is synthesized during vegetative growth (29), a fact consistent with the presence of a putative promoter having a −10 sequence identical to the consensus sequence recognized by the primary vegetative RNA polymerase 88 nt upstream of the ORF for the 125-kDa protein (Fig. 2). This promoter may also be recognized by the primary *E. coli* RNA polymerase, allowing its expression in this organism. In *E. coli*, the 125-kDa protein accumulated in the cell (Fig. 3) and was not exported into the medium, indicating that the leader peptide is not functional in this species. In *B. sphaericus*, the 122-kDa protein is associated

TABLE 1. Codon usage for the genes encoding the crystal proteins

| Amino acid | Codon | Gene product ($10^3$ mol wt) | | | $F_{Bsph}$[b] | $F_{Bsub}$[c] | $F_{Ecol}$[c] |
|---|---|---|---|---|---|---|---|
| | | 42[a] | 51[a] | 125 | | | |
| Phe | UUU | 16 | 16 | 11 | 0.55 | 0.57 | 0.37 |
| Phe | UUC | 3 | 5 | 27 | 0.45 | 0.43 | 0.63 |
| Leu | UUA | 11 | 16 | 53 | 0.60 | 0.24 | 0.07 |
| Leu | UUG | 4 | 6 | 0 | 0.07 | 0.14 | 0.09 |
| Leu | CUU | 3 | 7 | 13 | 0.17 | 0.25 | 0.07 |
| Leu | CUC | 1 | 1 | 0 | 0.01 | 0.10 | 0.07 |
| Leu | CUA | 2 | 8 | 8 | 0.13 | 0.06 | 0.02 |
| Leu | CUG | 1 | 0 | 0 | 0.01 | 0.21 | 0.68 |
| Ile | AUU | 12 | 15 | 39 | 0.52 | 0.50 | 0.36 |
| Ile | AUC | 8 | 4 | 20 | 0.25 | 0.37 | 0.61 |
| Ile | AUA | 13 | 14 | 2 | 0.23 | 0.13 | 0.03 |
| Met | AUG | 8 | 7 | 6 | 1.00 | 1.00 | 1.00 |
| Val | GUU | 8 | 9 | 79 | 0.56 | 0.31 | 0.36 |
| Val | GUC | 1 | 0 | 0 | 0.01 | 0.24 | 0.15 |
| Val | GUA | 5 | 11 | 49 | 0.38 | 0.21 | 0.22 |
| Val | GUG | 1 | 1 | 6 | 0.05 | 0.24 | 0.27 |
| Ser | UCU | 5 | 8 | 47 | 0.40 | 0.23 | 0.23 |
| Ser | UCC | 5 | 3 | 0 | 0.05 | 0.09 | 0.27 |
| Ser | UCA | 8 | 10 | 26 | 0.29 | 0.20 | 0.07 |
| Ser | UCG | 1 | 1 | 2 | 0.03 | 0.11 | 0.11 |
| Ser | AGU | 6 | 9 | 7 | 0.15 | 0.11 | 0.06 |
| Ser | AGC | 6 | 2 | 4 | 0.08 | 0.26 | 0.26 |
| Pro | CCU | 9 | 17 | 6 | 0.46 | 0.30 | 0.12 |
| Pro | CCC | 3 | 1 | 0 | 0.06 | 0.09 | 0.07 |
| Pro | CCA | 9 | 6 | 16 | 0.45 | 0.21 | 0.16 |
| Pro | CCG | 1 | 1 | 0 | 0.03 | 0.40 | 0.65 |
| Thr | ACU | 19 | 10 | 74 | 0.52 | 0.15 | 0.25 |
| Thr | ACC | 5 | 4 | 0 | 0.05 | 0.16 | 0.50 |
| Thr | ACA | 14 | 12 | 54 | 0.40 | 0.45 | 0.07 |
| Thr | ACG | 2 | 4 | 2 | 0.04 | 0.24 | 0.18 |
| Ala | GCU | 2 | 6 | 73 | 0.49 | 0.27 | 0.26 |
| Ala | GCC | 2 | 0 | 1 | 0.02 | 0.22 | 0.21 |
| Ala | GCA | 7 | 10 | 55 | 0.44 | 0.29 | 0.22 |
| Ala | GCG | 3 | 3 | 3 | 0.05 | 0.22 | 0.31 |
| Tyr | UAU | 18 | 21 | 6 | 0.62 | 0.65 | 0.40 |
| Tyr | UAC | 4 | 6 | 18 | 0.38 | 0.35 | 0.60 |
| His | CAU | 6 | 5 | 5 | 0.84 | 0.67 | 0.54 |
| His | CAC | 1 | 2 | 0 | 0.16 | 0.33 | 0.46 |
| Gln | CAA | 11 | 14 | 16 | 0.87 | 0.52 | 0.24 |
| Gln | CAG | 2 | 4 | 0 | 0.13 | 0.48 | 0.76 |
| Asn | AAU | 20 | 28 | 38 | 0.62 | 0.56 | 0.26 |
| Asn | AAC | 6 | 7 | 40 | 0.38 | 0.44 | 0.74 |
| Lys | AAA | 8 | 24 | 120 | 0.89 | 0.73 | 0.76 |
| Lys | AAG | 6 | 4 | 8 | 0.11 | 0.27 | 0.24 |
| Asp | GAU | 17 | 21 | 69 | 0.78 | 0.61 | 0.46 |
| Asp | GAC | 2 | 3 | 26 | 0.22 | 0.39 | 0.54 |
| Glu | GAA | 14 | 24 | 66 | 0.83 | 0.70 | 0.73 |
| Glu | GAG | 8 | 6 | 7 | 0.17 | 0.30 | 0.27 |
| Cys | UGU | 3 | 3 | 0 | 0.67 | 0.68 | 0.43 |
| Cys | UGC | 0 | 3 | 0 | 0.33 | 0.32 | 0.57 |
| Trp | UGG | 3 | 3 | 3 | 1.00 | 1.00 | 1.00 |
| Arg | CGU | 1 | 1 | 8 | 0.21 | 0.22 | 0.56 |
| Arg | CGC | 2 | 1 | 1 | 0.08 | 0.19 | 0.36 |
| Arg | CGA | 1 | 4 | 0 | 0.10 | 0.12 | 0.03 |
| Arg | CGG | 0 | 2 | 0 | 0.04 | 0.14 | 0.03 |
| Arg | AGA | 9 | 9 | 3 | 0.44 | 0.22 | 0.01 |
| Arg | AGG | 2 | 4 | 0 | 0.13 | 0.11 | 0.01 |
| Gly | GGU | 4 | 7 | 32 | 0.42 | 0.24 | 0.48 |
| Gly | GGC | 2 | 2 | 11 | 0.15 | 0.31 | 0.39 |
| Gly | GGA | 10 | 12 | 14 | 0.35 | 0.32 | 0.05 |
| Gly | GGG | 6 | 1 | 2 | 0.09 | 0.13 | 0.08 |
| Ter | UAA | 1 | 0 | 1 | 0.67 | 0.71 | 0.75 |
| Ter | UAG | 0 | 0 | 0 | 0.00 | 0.08 | 0.08 |
| Ter | UGA | 0 | 1 | 0 | 0.33 | 0.21 | 0.17 |

[a] From sequence in reference 3.
[b] Total codons for *B. sphaericus* 2362 = 1,997.
[c] *B. subtilis* and *E. coli* usage from reference 36.

with the cell wall (28, 29, 45; this study). Upon completion of exponential growth, there is a burst of synthesis of the 122-kDa protein by *B. sphaericus* (9), suggesting a complex pattern of regulation for the expression of gene 125. Adachi et al. (1) found that the expression of cell wall proteins of *B. brevis* 47 is regulated by three tandemly arranged promoters. Evidence was presented for differential utilization of these promoters during the growth and sporulation cycle of *B. brevis* 47.

The lack of toxicity for the larvae of *C. pipiens* of the cell wall-associated 122-kDa protein obtained from exponentially growing cells and the toxicity of the 110-kDa protein purified from the crystal preparation raise the possibility that the proteolytic degradation of the 122-kDa protein to the 110-kDa protein observed during sporulation (9) is a conversion of a protoxin to a toxin. Previous studies (33) with the related strain 1593 also indicated that vegetative cells were not toxic for mosquito larvae. The toxicity of cell wall preparations obtained from strain 1593 (32) could be the consequence of the presence of a 110-kDa protein since the cultures from which the preparations were obtained were in the early stationary phase, a time when some 110-kDa protein would be expected to be present (9). A similar interpretation may account for the "cytoplasmic toxin" of Davidson (12), which was found to have a molecular size of about 100 kDa. The absence of toxicity of *E. coli* cells containing proteins of 110 to 113 kDa derived from the 125-kDa protein (Fig. 3) may be due to the absence or difference in the pattern of glycosylation of these proteins compared with *B. sphaericus* or due to the removal of amino acids at the N terminus.

Amino acid sequence comparisons of the 122-kDa protein with the S-layer proteins from *Halobacterium halobium* (27) and *Deinococcus radiodurans* (35) revealed no primary sequence similarity. Comparisons with the sequence of the "outer wall protein" and "middle wall protein" of *B. brevis* 47 (42, 43), which form the S layer in this species, indicated that the 122-kDa protein had a significant sequence similarity to the N-terminal portion of the "outer wall protein" (Fig.

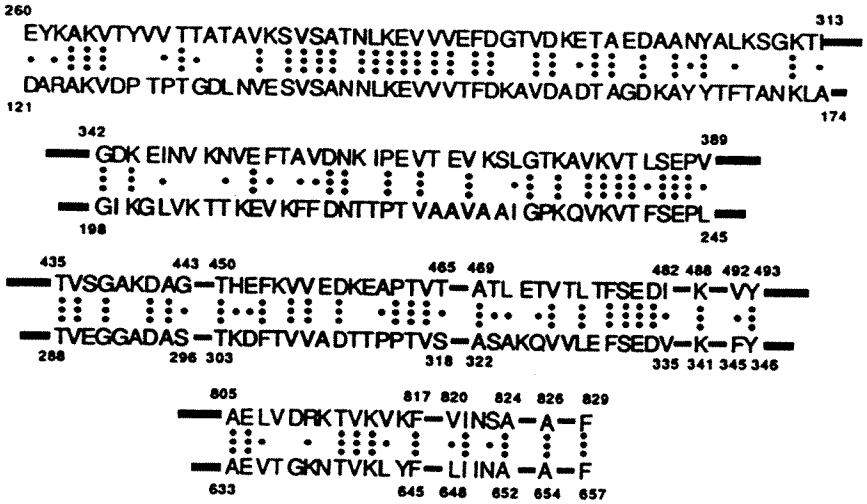



FIG. 9. Amino acid sequence similarity of the 122-kDa protein from *B. sphaericus* and the "outer wall protein" of *B. brevis* (43). The top sequence is from *B. sphaericus* 2362, and the bottom sequence is from *B. brevis* 47. Numbers indicate the position of the amino acids in the sequence. Three dots indicate identical amino acids, and single dots indicate conserved amino acid substitutions. Horizontal bars of unequal length represent adjustments necessary for alignment.

9). As for the 42- and 51-kDa proteins, the 122-kDa protein had no sequence similarity to any of the published Diptera-, Lepidoptera-, or Coleoptera-active toxins of *Bacillus thuringiensis* (8, 14, 20, 38, 44; and references in reference 3) or to the 42- and 51-kDa proteins from *B. sphaericus* 2362. All the published sequences of the *B. thuringiensis* lepidoptera- and diptera-active crystal proteins have some sequence homology, suggesting a common evolutionary origin. In contrast, the 122-kDa protein of *B. sphaericus* is related to at least one S-layer protein, while the related 42- and 51-kDa crystal proteins (3) appear to be distinct. The putative 80-kDa protein coded for by a cryptic gene is highly related to a major portion of the 122-kDa protein, suggesting a previous gene duplication (Fig. 8). Evidence for a cryptic gene having a sequence related to the Lepidoptera-active crystal proteins from *B. thuringiensis* has been previously presented (24).

The present study completes the genetic identification and characterization of the genes of *B. sphaericus* coding for proteins which had been previously found to be toxic for mosquito larvae (4, 7, 9, 12, 47). In our first publication on the crystal proteins of *B. sphaericus* 2362, we obtained antisera to the 42- and 51-kDa proteins and using these antisera detected a cross-reaction with the 122- and 110-kDa proteins in Western immunoblots (4). In view of the lack of sequence similarity between the low- and high-molecular-weight proteins revealed in this study, these results can only be interpreted as indicating that the 42- and 51-kDa proteins originally purified from the crystal preparation and used to raise antisera contained degradation products of the 122- and 110-kDa proteins similar in molecular size to the 42- and 51-kDa proteins.

## ACKNOWLEDGMENTS

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